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(54) Title: DUAL RECOMBINANT GENE THERAPY COMPOSITIONS AND METHODS OF USE

(57) Abstract: The present invention relates to novel compositions and methods for the treatment of cardiovascular disease. More particularly, the invention relates to gene therapy compositions comprising at least two transgenes encoding angiogenic proteins or peptides. In one aspect the two transgenes are provided in a single gene delivery vector. Alternatively, the composition comprises at least two vectors, each vector comprising a transgene encoding a different angiogenic protein or peptide. The invention also relates to methods of treating cardiovascular disease using the gene therapy compositions; kits for gene delivery; and pharmaceutical compositions.

DUAL RECOMBINANT GENE THERAPY COMPOSITIONS AND METHODS OF USE

FIELD OF THE INVENTION

The present invention relates to novel compositions and methods for their use in treating disease. In particular, presented herein are novel angiogenic gene therapy compositions and methods for their use to treat cardiovascular disease.

BACKGROUND OF THE INVENTION

Cardiovascular disease is a major worldwide health problem. In the United States, for example, approximately 1 of every 5 people, nearly 60 million in total, is presently afflicted with some form of cardiovascular disease. Cardiovascular disease is also the single largest cause of death in the United States, accounting for about 40% of all deaths each year. According to the American Heart Association, it is estimated that about 6 million people suffer from angina pectoris, a common condition of coronary artery disease, and approximately 4.6 million suffer from congestive heart failure, a manifestation of heart disease that, in 50% of diagnosed cases, results in death within 5 years of diagnosis. Additionally, it has been estimated that over 700,000 people suffer from peripheral vascular disease, of whom over 100,000 may require a limb amputation.

The statistics for cardiovascular disease are similarly grim in many other countries; and, in many cases, the problem of cardiovascular disease has become increasingly more apparent as life spans have increased.

Due to the importance of the cardiovascular system in maintaining systemic health, cardiovascular disease can be extremely debilitating, as well as deadly. For example, one manifestation of heart disease, angina pectoris, is characterized by transient periods of myocardial ischemia (i.e., inadequate blood supply to the heart muscle or myocardium) resulting in chest pain. For some patients, such chest pains may occur sporadically and mildly, but for other patients, the pain is severe and frequent, such that normal activities, such as walking for more than a few minutes, are painful and/or impossible.

In addition to angina, myocardial ischemia may result in such heart diseases as heart attack and/or congestive heart failure ("CHF"). Most commonly, atherosclerosis (also known as coronary artery disease or "CAD") is at the root of myocardial ischemia. Atherosclerosis is typically caused by calcification and plaque formation on the interior surface of a blood vessel, which reduces blood flow to the myocardium. For the patient suffering from myocardial ischemia, a heart attack can occur when the coronary artery disease is so severe that one or more arteries are completely or nearly completely blocked. Frequently, such heart attacks result in cell death or necrosis of heart tissue in the region supplied by the diseased artery and thus, permanent heart damage. For some myocardial ischemia patients, particularly those with such severe myocardial ischemia that they have experienced a heart attack, a condition known as congestive heart failure (CHF) results. CHF is defined as abnormal heart function resulting in inadequate cardiac output to meet metabolic needs. (Braunwald, E. (ed), In: Heart Disease, W.B. Saunders, Philadelphia, page 426, 1988.) Although severe myocardial ischemia is the most common cause of CHF, the condition can also arise as a result of other injuries to the heart. CHF is generally classified, according to the New York Heart Association (NYHA) Functional Classification system, on a scale of Class I-IV, in terms of increasing severity. Since the heart of a patient with even moderately severe CHF (e.g., Class III) is in a constant struggle to function adequately to meet even the most basic needs of the patient, the prognosis for such patients is dismal, with only half surviving more than two years following diagnosis.

Unfortunately, despite the advances made in understanding cardiovascular diseases such as heart disease, presently available therapies are often inadequate and the costs of these diseases, both in terms of dollars and in terms of quality and quantity of human life, are very high. Presently, treatments for heart disease include pharmacological therapies, coronary revascularization procedures (such as angioplasty and bypass surgery) and, in the most severe cases, heart transplant. With respect to pharmacological therapies, the goal for treating myocardial ischemia has been to increase the blood supply to the heart muscle and/or to decrease the demand of the heart muscle for oxygen and nutrients. For example, agents such as calcium

channel blockers or nitroglycerin may be employed to increase blood supply; arterial vasodilators may be employed to decrease the hemodynamic load on the heart and/or beta-adrenergic receptor antagonists may be used to decrease the contractile response of the heart to a given hemodynamic load. With respect to treatment of congestive heart failure, pharmacologics such as digitalis or beta-adrenergic receptor agonists may be used to increase the force of contraction of the heart, diuretics may be used to reduce fluid accumulation in the lungs and elsewhere, and/or angiotensin converting enzyme ("ACE") inhibitors, or similar pharmacologics that decrease systemic vascular resistance, may be used to reduce the work of the heart. While the pharmacological therapies presently employed to treat CHF can improve some of the symptoms of the disease and potentially prolong life, the overall prognosis for these patients remains extremely poor.

Some heart disease patients can benefit from revascularization procedures, such as coronary artery bypass graft (CABG) surgery or balloon angioplasty, such as percutaneous transluminal coronary angioplasty. Generally, those patients that suffer from coronary artery disease that has not yet resulted in heart tissue necrosis can benefit the most from such procedures. If, however, the patient has an inadequate microvascular bed (such as, may be found in more severe CHF patients), revascularization will rarely restore cardiac function to normal or near-normal levels. Further, even in those patients most likely to benefit from such procedures, complications such as failure of the bypass graft(s) or restenosis following angioplasty present further risk and limit the overall benefits of these procedures. Given the particularly poor prognosis for patients with congestive heart failure, heart transplant surgery is sometimes the only hope for prolonged survival. However, heart transplant procedures are generally only available to CHF patients who have no other confounding diseases and are relatively young. Moreover, the very small supply of transplantable hearts and the extreme cost of transplant surgery are significant limiting factors in the applicability of this particular therapy.

As with heart disease, other cardiovascular diseases, such as peripheral vascular diseases, often result from inadequate blood flow to tissue which then becomes ischemic. By way of illustration, peripheral arterial vascular disease (PVD)

results from atherosclerosis in a peripheral vessel which then may cause ischemia in the tissue supplied by that vessel. The most common form of PVD is peripheral arterial occlusive disease. As with other cardiovascular diseases, peripheral vascular diseases are generally treated with pharmacologics, such as, aspirin or other agents that reduce the viscosity of blood, or by surgical interventions, such as angioplasty, vessel grafting and/or removal of fatty deposits. Although the available treatments for peripheral vascular diseases may ameliorate symptoms, their overall effectiveness is typically inadequate, for reasons similar to those discussed above with respect to heart diseases.

There is thus a substantial continuing need for compositions and methods for the treatment of cardiovascular diseases to improve the prognosis for patients suffering from this major health problem.

SUMMARY OF THE INVENTION

The present invention is directed to novel methods and compositions for the treatment of disease. More particularly, the invention relates to compositions that promote angiogenesis and to the use of those compositions to treat cardiovascular disease. Still more particularly, combinations of genes encoding angiogenic proteins or peptides are employed in *in vivo* methods to prevent, ameliorate and/or treat cardiovascular disease, such as heart disease and peripheral vascular disease.

In one aspect, the present invention provides methods of treating a patient suffering from a cardiovascular disease comprising delivering to the patient a vector comprising at least two transgenes encoding angiogenic proteins or peptides, wherein said transgenes are expressed in said patient. In preferred embodiments, the transgenes each encode a different angiogenic protein or peptide, and most preferably the transgenes are from different angiogenic gene families. Preferably, delivery of the compositions according to the present invention is targeted to a region of tissue and facilitates or stimulates angiogenesis in that tissue.

In preferred embodiments, methods are provided of promoting angiogenesis in a patient comprising delivering to a region of tissue, such as an ischemic tissue, by

introducing the vector into a vessel (or conduit) connected to said region, wherein the vector comprises at least two transgenes encoding angiogenic proteins or peptides and is capable of expressing said transgenes in the patient. By way of illustration, in a preferred embodiment for the treatment of heart disease, methods are provided of stimulating angiogenesis in a patient comprising delivering to the myocardium of the patient by intracoronary injection directly into one or more coronary arteries, a vector comprising at least two transgenes, each encoding an angiogenic protein or peptide, wherein the vector is capable of expressing the angiogenic proteins or peptides in the myocardium.

In other preferred embodiments, a method is provided for stimulating vessel development in a patient having peripheral vascular disease, comprising delivering a vector to the peripheral vascular system of the patient, such as by intramuscular injection into the affected tissue or by intra-arterial injection directly into one or more peripheral arteries (such as femoral arteries supplying the leg), said vector comprising at least two transgenes encoding angiogenic proteins or peptides, wherein said transgenes express the angiogenic proteins or peptides in the peripheral vascular tissue, thereby promoting peripheral vascular development.

In one aspect, the methods and compositions of the present invention employ a single vector, preferably a viral vector, such as an adenoviral or adeno-associated viral (AAV) vector, comprising at least two transgenes encoding angiogenic proteins or peptides. Alternatively, the combination of transgenes can be provided using multiple vectors. By way of illustration, a pair of adenoviral vectors may be used, each comprising a different angiogenic transgene. Different vectors may also be employed. For example, an adenoviral vector comprising an angiogenic transgene may be used in combination with an AAV or other viral or non-viral vector comprising another angiogenic gene.

Preferably, the angiogenic proteins or peptides encoded by the combination of at least two transgenes are different from one another. More preferably, the transgenes are from different angiogenic gene families. By way of illustration, in particularly preferred embodiments, one transgene encodes a member of the fibroblast

growth factor (FGF) family and a second transgene encodes a member of the vascular endothelial growth factor (VEGF) family.

Additional aspects of the invention include an injectable preparation comprising a recombinant vector (or vectors) comprising at least two transgenes encoding angiogenic proteins or peptides. In a particularly preferred embodiment, the injectable preparation comprises: a recombinant adenoviral vector, said vector being replication deficient and comprising a partial adenoviral sequence (e.g. a sequence from which the E1A/E1B genes have been deleted), and at least two transgenes coding for angiogenic proteins or peptides (which may be driven by one or more promoters); and a pharmaceutically suitable carrier.

Additional aspects of the present invention include kits for gene delivery comprising a composition selected from the group consisting of: (i) a composition comprising a vector which comprises at least two transgenes encoding angiogenic proteins or peptides; (ii) a composition comprising at least two vectors, each of which comprises a transgene encoding a different angiogenic protein or peptide; and (iii) a composition comprising at least two different vectors, each of which comprises a transgene encoding an angiogenic protein or peptide. In one embodiment the kit further comprises a device for introducing the composition into a blood vessel or tissue *in vivo*, preferably a catheter (such as a catheter for infusion into a coronary artery or a peripheral artery, or for injection into a peripheral muscular tissue, as applicable). In another embodiment, the kit includes a vasoactive agent (such as histamine, a histamine agonist, a VEGF, or a nitric oxide donor (e.g. sodium nitroprusside)).

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

An "angiogenic protein or peptide" refers to any protein or peptide capable of inducing or promoting angiogenesis or angiogenic activity, i.e. blood vessel development and/or differentiation, either directly or indirectly, such as, for example, by enhancing the expression, stability or functionality of other angiogenic proteins.

Various angiogenic proteins are known and new ones are routinely identified. By way of illustration, angiogenic proteins or peptides include, without limitation: fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) (including vascular endothelial growth factor-related proteins (VRPs)), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), angiopoietins (Angs) hepatocyte growth factor (also known as scatter factor), granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), transforming growth factor-beta (TGF-beta), hypoxia-inducible factors (Hifs) and angiogenic zinc finger proteins (AZFPs). Additionally, analogs and derivatives of angiogenic proteins or peptides, having angiogenic activity, are likewise considered "angiogenic proteins or peptides", herein.

A "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. This term refers to the primary structure of the molecule, and thus includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modified polynucleotides such as methylated and/or capped polynucleotides.

"Recombinant," as applied to a polynucleotide, means that the polynucleotide is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in a polynucleotide construct that is distinct from a polynucleotide found in nature.

"Gene" and "transgene" (i.e. a transferred gene or a gene to be transferred) each refer to a polynucleotide or portion of a polynucleotide comprising a nucleotide sequence that encodes a protein or peptide. For most situations, it is desirable for a gene to further comprise a promoter operably linked to the coding sequence or sequences in order to be effectively expressed. Enhancers, repressors and other regulatory sequences may also be included within the gene in order to modulate activity of the gene, as is well known in the art. (See, e.g., the references cited below). "Angiogenic transgene" as used herein refers to a transgene that encodes an angiogenic protein or peptide.

The terms "polypeptide," "peptide," and "protein" are used interchangeably to refer to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include glycosylation, acetylation and phosphorylation.

A "heterologous" component refers to a component that is introduced into or produced within a different entity from that in which it is naturally located. For example, a polynucleotide derived from one organism and introduced by genetic engineering techniques into a different organism is a heterologous polynucleotide which, if expressed, can encode a heterologous polypeptide. Similarly, a promoter or enhancer that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous promoter or enhancer.

A "promoter," as used herein, refers to a polynucleotide sequence that controls transcription of a coding sequence to which it is operably linked. A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources, are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources).

An "enhancer," as used herein, refers to a polynucleotide sequence that enhances transcription of a coding sequence to which it is operably linked. A large number of enhancers, from a variety of different sources are well known in the art (and identified in databases such as GenBank) and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences. Thus, as used herein, the term "promoter" also includes any enhancers associated therewith.

A "transcription regulator" or "regulatory sequence" refers to a polynucleotide sequence that controls, enhances or otherwise affects transcription of a gene (or transgene) to which it is operably linked. Examples of such regulatory sequences

include, without limitation, promoters, enhancers, polyadenylation sequences and the like.

"Operably linked" refers to a juxtaposition of two or more components, wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter is operably linked to a coding sequence if the promoter controls transcription of the coding sequence. Although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within, or downstream of coding sequences. A polyadenylation sequence is operably linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence.

A "replicon" refers to a polynucleotide comprising an origin of replication which allows for replication of the polynucleotide in an appropriate host cell. Examples include chromosomes of a target cell into which a heterologous nucleic acid might be integrated (e.g., nuclear and mitochondrial chromosomes), as well as extrachromosomal replicons (such as replicating plasmids and episomes).

"Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stable or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of

vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

"*In vivo*" gene delivery, gene transfer, gene therapy and the like, as used herein, are terms referring to the introduction of an exogenous polynucleotide (which may or may not be a heterologous polynucleotide), for example, contained in a gene delivery vector, directly into the body of an organism, such as a human or non-human mammal, whereby the exogenous polynucleotide is introduced into a cell of such organism *in vivo*.

A "vector" (sometimes referred to as a gene delivery or gene transfer vector or "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered may comprise one or more coding sequences of interest as well as additional polynucleotide sequences that may, for example, enhance transfer, integration, or expression.

"Vasculature" or "vascular" are terms referring to the system of vessels carrying blood (as well as lymph fluids) throughout the mammalian body.

"Blood vessel" refers to any of the vessels of the mammalian vascular system, including arteries, arterioles, capillaries, venules, veins, sinuses and vasa vasorum. In preferred aspects of the present invention for treating heart disease, vectors comprising angiogenic transgenes are introduced directly into vascular conduits supplying blood to the myocardium. Such vascular conduits include the coronary arteries as well as vessels such as saphenous veins or internal mammary artery grafts.

"Artery" refers to a blood vessel through which blood passes away from the heart. Coronary arteries supply the tissues of the heart itself (particularly the myocardium), while other arteries supply the remaining organs of the body. The general structure of an artery consists of a lumen surrounded by a multi-layered arterial wall.

An "individual" or a "patient" refers to a mammal, preferably a large mammal, most preferably a human.

"Treatment" or "therapy" as used herein refers to administering agents to an individual patient that are capable of eliciting a prophylactic, ameliorative, curative or other beneficial effect on the individual.

As used herein "gene transfer" means the process of introducing a nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid molecule contact with a target cell by non-specific or receptor-mediated interactions, uptake of nucleic acid molecules into the cell through the membrane or by endocytosis, and release of nucleic acid molecule into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid molecule into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell *in vivo* or *in vitro*. Gene transfer can be performed ex vivo on cells which are then transplanted into a patient, or can be performed by direct administration of the nucleic acid molecule (or associated complex) into the patient.

A "therapeutic polynucleotide" or "therapeutic gene" refers to a nucleotide sequence that is capable, when transferred to an individual, of eliciting a prophylactic, ameliorative, curative or other beneficial effect in the individual.

"Heart disease" refers to acute and/or chronic cardiac dysfunctions. Heart disease is often associated with a decrease in cardiac contractile function and may be associated with an observable decrease in blood flow to the myocardium (e.g., as a

result of coronary artery disease). Manifestations of heart disease include myocardial ischemia, which may result in angina, heart attack and/or congestive heart failure.

"Myocardial ischemia" is a condition in which the heart muscle does not receive adequate levels of oxygen and nutrients relative to physiological needs, which is typically due to inadequate blood supply to the myocardium (e.g., as a result of coronary artery disease).

"Heart failure" is clinically defined as a condition in which the heart does not provide adequate blood flow to the body to meet metabolic demands. Symptoms include breathlessness, fatigue, weakness, leg swelling, and exercise intolerance. On physical examination, patients with heart failure tend to have elevations in heart and respiratory rates, rales (an indication of fluid in the lungs), edema, jugular venous distension, and, in many cases, enlarged or "dilated" hearts. Patients with severe heart failure suffer a high mortality; typically 50% of the patients die within two years of developing the condition. In some cases, heart failure is associated with severe coronary artery disease ("CAD"), typically resulting in myocardial infarction and either progressive chronic heart failure or an acute low output state, as described herein and in the art. In other cases, heart failure is associated with dilated cardiomyopathy without associated severe coronary artery disease.

"Peripheral vascular disease" or "PVD" refers to acute or chronic dysfunction of the peripheral (*i.e.*, non-cardiac) vasculature and/or the tissues supplied thereby. As with heart disease, peripheral vascular disease typically results from an inadequate blood flow to the tissues supplied by the vasculature, whereby the lack of blood may further result, for example, in ischemia or, in severe cases, in tissue cell death. Frequently, symptoms of peripheral vascular disease are manifest in the extremities of the patient, especially the legs.

As used herein, the terms "having therapeutic effect" and "successful treatment" carry essentially the same meaning. In particular, a patient suffering from heart disease is successfully "treated" for the condition if the patient shows observable and/or measurable reduction in or absence of one or more of the symptoms of heart

disease. Reduction of these signs or symptoms may also be felt by the patient. Thus, for example, indicators of successful treatment of heart disease conditions include the patient showing or feeling a reduction in any one of the symptoms of angina pectoris, fatigue, weakness, breathlessness, leg swelling, rales, heart or respiratory rates, edema or jugular venous distension. The patient may also show greater exercise tolerance, have a smaller heart with improved ventricular and cardiac function, and in general, require fewer hospital visits related to the heart condition. The improvement in cardiovascular function may be adequate to meet the metabolic needs of the patient and the patient may not exhibit symptoms under mild exertion or at rest. Many of these signs and symptoms are readily observable by eye and/or measurable by routine procedures familiar to a physician. Indicators of improved cardiovascular function include increased blood flow and/or contractile function in the treated tissues. Blood flow in a patient can be measured, for example, by thallium imaging (as described by Braunwald in Heart Disease, 4th ed., pp. 276-311 (Saunders, Philadelphia, 1992)) or by echocardiography (described in Sahn, DJ, et al., *Circulation.* 58:1072-1083, 1978). Blood flow before and after angiogenic gene transfer can be compared using these methods. Improved heart function is generally associated with decreased signs and symptoms, as noted above. In addition to echocardiography, one can measure ejection fraction (EF), (e.g. from the left ventricle) by nuclear and other techniques as are known in the art. Blood flow and contractile function can likewise be measured in peripheral tissues treated according to the present invention.

References

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained in the literature. See e.g., Molecular Cloning: A Laboratory Manual, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); Current Protocols in Molecular Biology (F. Ausubel et al. eds., 1987 and updated); Essential Molecular Biology (T. Brown ed., IRL Press 1991); Gene Expression Technology (Goeddel ed., Academic Press 1991); Methods for Cloning and Analysis of Eukaryotic Genes (A. Bothwell et al. eds., Bartlett Publ. 1990); Gene Transfer and Expression (M. Kriegler, Stockton Press

1990); Recombinant DNA Methodology (R. Wu et al. eds., Academic Press 1989); PCR: A Practical Approach (M. McPherson et al., IRL Press at Oxford University Press 1991); Cell Culture for Biochemists (R. Adams ed., Elsevier Science Publishers 1990); Gene Transfer Vectors for Mammalian Cells (J. Miller & M. Calos eds., 1987); Mammalian Cell Biotechnology (M. Butler ed., 1991); Animal Cell Culture (J. Pollard et al. eds., Humana Press 1990); Culture of Animal Cells, 2nd Ed. (R. Freshney et al. eds., Alan R. Liss 1987); Flow Cytometry and Sorting (M. Melamed et al. eds., Wiley-Liss 1990); the series Methods in Enzymology (Academic Press, Inc.); Techniques in Immunocytochemistry, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); Cellular and Molecular Immunology (A. Abbas et al., W.B. Saunders Co. 1991, 1994); Current Protocols in Immunology (J. Coligan et al. eds. 1991); the series Annual Review of Immunology; the series Advances in Immunology; Oligonucleotide Synthesis (M. Gait ed., 1984); Animal Cell Culture (R. Freshney ed., IRL Press 1987); the series Arteriosclerosis, Thrombosis and Vascular Biology (Lippincott, Williams & Wilkins publishers for the American Heart Association); the series Circulation (Lippincott, Williams & Wilkins publishers for the American Heart Association); and the series Circulation Research (Lippincott, Williams & Wilkins publishers for the American Heart Association).

Additional references describing delivery and logistics of surgery which may be used in the methods of the present invention include the following: Topol, EJ (ed.), The Textbook of Interventional Cardiology, 2nd Ed. (W.B. Saunders Co. 1994); Rutherford, RB, Vascular Surgery, 3rd Ed. (W.B. Saunders Co. 1989); The Cecil Textbook of Medicine, 19th Ed. (W.B. 1992); and Sabiston, D, The Textbook of Surgery, 14th Ed. (W.B. 1991). Additional references describing cell types found in the blood vessels, and those of the vasculature which may be useful in the methods of the present invention include the following: W. Bloom & D. Fawcett, A Textbook of Histology (V.B. Saunders Co. 1975).

Various publications have postulated on the uses of gene transfer for the prevention of disease, including heart disease. See, e.g., Methods in Virology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Weiss, Clifton, N.J., 1991;

Mazur et al., Molecular and Cellular Biology, 21:104-111, 1994; French, Herz 18:222-229, 1993; Williams, Journal of Medical Sciences 306:129-136, 1993; and Schneider, Circulation 88:1937-1942, 1993.

Similarly, various publications describe various vectors that may be useful in gene therapy methods. See, e.g., Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679-1721, (1990); Graham, F., et al., pp. 109-128 in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., FASEB Journal 9:190-199, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68:145-159, 1994; Schneider and French, Circulation 88:1937-1942, 1993; Curiel D.T., et al., Human Gene Therapy 3:147-154, 1992; Graham, F.L., et al., WO 95/00655, published 5 Jan. 1995; Falck-Pedersen, E.S., WO 95/16772, published 22 Jun. 1995; Denefle, P. et al., WO 95/23867, published 8 Sep. 1995; Haddada, H. et al., WO 94/26914, published 24 Nov. 1994; Perricaudet, M. et al., WO 95/02697, published 26 Jan. 1995; Zhang, W., et al., WO 95/25071, published 12 Oct. 1995; Hammond, et al., WO 96/26742, published 6 Sep. 1996; Hammond et al., USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by In Vivo Gene Delivery", hereby incorporated by reference in its entirety; Gnatenko, D., J. of Invest. Med. 45:87-97, (1997); Carter, B., Handbook of Parvoviruses, vol. I, pp. 169-228, 1990; Berns, Virology, pp. 1743-1764 (Raven Press 1990); Carter, B., Curr. Opin. Biotechnol., 3:533-539, 1992; Muzyczka, N., Current Topics in Microbiology and Immunology, 158:92-129, 1992; Flotte, T.R., et al., Am. J. Respir. Cell Mol. Biol. 7:349-356, 1992; Chatterjee et al., Ann. NY Acad. Sci., 770: 79-90, 1995; Flotte, T.R., et al., WO 95/13365, published 18 May 1995; Trempe, J.P., et al., WO 95/13392, published 18 May 1995; Kotin, R., Human Gene Therapy, 5: 793-801, 1994; Flotte, T.R., et al., Gene Therapy 2:357-362, 1995; Allen, J.M., WO 96/17947, published 13 Jun. 1996; and Du et al., Gene Therapy 3: 254-261, 1996.

Incorporation By Reference

All references cited within this application, including patents, patent applications and other publications, are hereby incorporated by reference.

DETAILED DESCRIPTION OF VARIOUS PREFERRED EMBODIMENTS

Various preferred aspects of the present invention are summarized below and further detailed and illustrated in the subsequent Examples and Figures.

The present invention features methods and compositions for the treatment of cardiovascular disease. In particular, provided herein are methods and compositions employing at least two angiogenic proteins or peptides in the *in vivo* treatment of cardiovascular disease. In preferred aspects, the present invention provides methods of treating cardiovascular disease comprising delivering, preferably in a tissue-targeted manner, a vector or combination of vectors to a patient, which vector or vectors collectively comprise at least two transgenes encoding angiogenic proteins or peptides, and expressing said transgenes *in vivo*. Most preferably, the angiogenic transgenes employed in the present methods and compositions are selected to be from two different angiogenic gene families, illustrative examples of which are described herein.

Gene Therapy

In one preferred aspect, the present invention provides gene therapy methods to treat cardiovascular disease. Recent advances have resulted in practical approaches to human gene therapy that have demonstrated positive initial results. See, for example, the gene therapy techniques described by Hammond, et al. in WO 96/56742, published 6 Sep 1996; WO 98/10085, published 5 Sep. 1997; and WO 98/50079, published 12 Nov. 1998; USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by In Vivo Gene Delivery" hereby incorporated by reference in its entirety; and by Giordano, et al., Nat. Med., 2(5):534-9 (1996), which demonstrate the treatment of cardiovascular diseases by *in vivo* gene delivery. Use of an appropriate preclinical model is an important aspect of

developing therapies that are capable of being effectively used in human patients. As reported by Hammond et al., the porcine ischemia model has proven to be an important component of developing cardiovascular gene therapies for conditions such as myocardial ischemia and/or congestive heart failure.

In a particularly preferred embodiment, a gene therapy composition comprising a vector containing at least two transgenes encoding angiogenic proteins or peptides, is administered to a patient, whereby the transgenes are expressed in the patient. Preferably the gene therapy compositions are targeted to the tissue to be treated; for example, by delivering the composition into a conduit (i.e., vessel) associated with the target tissue or by direct injection into the tissue. Detailed examples illustrating the use of vectors comprising two transgenes encoding angiogenic proteins or peptides according to the present invention and results thereof are provided in the Examples below.

Various types of vectors known to those of skill in the art may be used for delivery of the combined angiogenic transgenes to a targeted cell population. Preferred are recombinant vectors derived from viruses. Examples of such viruses include, without limitation, adenovirus, adeno-associated virus, retroviruses, (e.g. lentivirus, feline immunodeficiency virus), vaccinia virus, herpes viruses, various RNA viruses and bovine papilloma virus. Additionally, other viruses useful as gene delivery vectors are regularly being developed and may likewise be employed in the context of the present invention. Methods of construction of recombinant viral vectors containing coding sequences are well known to those skilled in the art and may be used to construct the vectors containing angiogenic transgenes as described herein.

In exemplary embodiments, the vectors employed as described herein are recombinant adenoviral vectors comprising combined transgenes. References describing adenovirus vectors, as well as other viral vectors that can be used in the methods and compositions of the present invention, include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679-1721, (1990); Graham, F., et al., pp. 109-128 in

Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., FASEB Journal 9:190-199, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68:145-159, 1994; Schneider and French, Circulation 88:1937-1942, 1993; Curiel D.T., et al., Human Gene Therapy 3:147-154, 1992; Graham, F.L., et al., WO 95/00655, published 5 Jan. 1995; Falck-Pedersen, E.S., WO 95/16772, published 22 Jun. 1995; Denefle, P. et al., WO 95/23867, published 8 Sep. 1995; Haddada, H. et al., WO 94/26914, published 24 Nov. 1994; Perricaudet, M. et al., WO 95/02697, published 26 Jan. 1995; Zhang, W., et al., WO 95/25071, published 12 Oct. 1995; and Hammond, et al., WO 96/26742, published 6 Sep. 1996 and Hammond, et al., USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by In Vivo Gene Delivery", previously incorporated herein by reference. A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996).

In addition to adenoviral vectors, adeno-associated viral (AAV) vectors have been shown useful for gene delivery and likewise can be used in the present compositions and methods. (See, e.g., Gnatenko, D., J. of Invest. Med. 45:87-97, (1997) concerning use of AAV vectors to deliver genes into vascular cells). References describing AAV vectors include, without limitation, the following: Carter, B., Handbook of Parvoviruses, vol. I, pp. 169-228, 1990; Berns, Virology, pp. 1743-1764 (Raven Press 1990); Carter, B., Curr. Opin. Biotechnol., 3:533-539, 1992; Muzyczka, N., Current Topics in Microbiology and Immunology, 158:92-129, 1992; Flotte, T.R., et al., Am. J. Respir. Cell Mol. Biol. 7:349-356, 1992; Chatterjee et al., Ann. NY Acad. Sci., 770: 79-90, 1995; Flotte, T.R., et al., WO 95/13365, published 18 May 1995; Trempe, J.P., et al., WO 95/13392, published 18 May 1995; Kotin, R., Human Gene Therapy, 5: 793-801, 1994; Flotte, T.R., et al., Gene Therapy 2:357-362, 1995; Allen, J.M., WO 96/17947, published 13 Jun. 1996; and Du et al., Gene Therapy 3: 254-261, 1996.

In alternative methods, described further herein, a combination of adenoviral and adeno-associated viral vectors are employed, each comprising at least one

transgene encoding an angiogenic protein or peptide. In a similar manner, other vectors may be combined for use in gene therapy, according to the present invention. Use of combinations of vectors to deliver transgenes encoding different angiogenic proteins or peptides can provide various advantages such as, for example, permitting differential expression of the transgenes by selecting one vector for its ability to provide prolonged steady-level gene expression and selecting a second vector for its ability to provide shorter duration, higher level gene expression. By way of illustration, an adenovirus vector may be used to provide a relative "burst" of expression in conjunction with an AAV vector that generally provides for more steady and longer-lived expression.

In addition to viral vectors, numerous non-viral vectors are known to those of skill in the art that can be used to deliver combined transgenes in accordance with the present invention. References describing such non-viral vectors include, without limitation, the following: Ledley, FD, Human Gene Therapy 6:1129-1144, 1995; Miller, N., et al., FASEB Journal 9:190-199, 1995; Chonn, A., et al., Curr. Opin. in Biotech. 6:698-708, 1995; Schofield, JP, et al., British Med. Bull. 51:56-71, 1995; Brigham, K.L., et al., J. Liposome Res. 3:31-49, 1993; Philip, R., et al., Mol. Cell Biol. 14: 2411-2418, 1994; Brigham, K.L., WO 91/06309 (16 May 1991); Felgner, P.L., et al., WO 91/17424 (14 November 1991); Solodin et al., Biochemistry 34:13537-13544, 1995; WO 93/19768 (14 October 1993); Debs et al., WO 93/25673; Felgner, P.L., et al., U.S. Patent 5,264,618 (November 23, 1993); Epand, R.M., et al., U.S. Patent 5,283,185 (February 1, 1994); Gebeyehu et al., U.S. Patent 5,334,761 (August 2, 1994); Felgner, P.L., et al., U.S. Patent 5,459,127 (October 17, 1995); Overell, R.W., et al., WO 95/28494 (26 October 1995); Jessee, WO 95/02698 (26 January 1995); Haces and Ciccarone, WO 95/17373 (29 June 1995); Lin et al., WO 96/01840 (25 January 1996).

Other methods have also been described for introducing DNA into large numbers of cells. These methods include, for example: transfection, wherein DNA is precipitated with calcium phosphate and taken into cells by pinocytosis (Chen C. and Okayama H, Mol. Cell Biol. 7:2745-52 (1987)); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane to facilitate uptake of DNA (Chu G. et al., Nucleic Acids Res., 15:1311-26 (1987)); and particle

bombardment using DNA bound to small projectiles (Yang NS. et al., Proc. Natl. Acad. Sci. 87:9568-72 (1990)). Another method for introducing DNA into cells is to couple the DNA to chemically-modified proteins. Generally, these methods of gene transfer are performed outside of the body (i.e. *ex vivo*). Thus, for *ex vivo* gene therapy, cells of interest may be explanted from the patient and/or a non-diseased individual, treated with the transgenes, grown *in vitro* and then injected, infused, transplanted or otherwise introduced into the patient.

It has also been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents can substantially improve the uptake and expression of the recombinant gene. See, e.g., Curiel D., et al., Human Gene Therapy 3:147-154 (1992).

In another preferred embodiment, the gene therapy composition employs one or more vectors, comprising transgenes encoding angiogenic proteins or peptides, the nucleic acid molecule sequences of which vectors are expressed only in a specific tissue. Thus, in these embodiments, in addition (or alternatively) to physically targeting delivery of the vector composition to a specific region of tissue, the vector is further targeted such that it is preferentially expressed in specific tissue(s). Methods of achieving tissue-specific gene expression include, for example, use of tissue-tropic vectors, tissue-specific promoters and/or tissue-specific enhancers to selectively drive expression of the angiogenic transgenes. See, e.g., Franz, et al, Cir. Res., 73(4):629-38 (1993) and other references cited above.

Transgenes Encoding Angiogenic Proteins or Peptides

The compositions and methods of the present invention employ combinations of angiogenic proteins or peptides to treat cardiovascular disease. In preferred embodiments the compositions comprise at least two transgenes encoding angiogenic proteins or peptides, which transgenes are contained in one or more vectors. Preferably, the angiogenic proteins or peptides are different from one another and

most preferably are from different angiogenic protein families. Families of angiogenic genes include individual genes that are relatively closely related in terms of function and may share structural similarities as well (the families of FGFs and VEGFs being exemplary illustrations in those regards). Any protein or peptide that can exhibit angiogenic activity, enhance blood flow and/or contractile function, measurable by the methods described herein and in the art, can be potentially employed in connection with the present invention. Exemplary angiogenic genes that can be employed in the context of the present invention are illustrated herein.

As is well known to those of skill in the art, and as is fairly typical of many biological systems, there are numerous points at which multi-step pathways, such as angiogenesis, can be triggered and/or enhanced. For example, in the case of angiogenesis, many angiogenic proteins mediate or promote an angiogenic response by stimulating or potentiating other angiogenic proteins (or, to similar effect, by inhibiting or deactivating a repressor of an angiogenic protein). By way of illustration, fibroblast growth factors (FGFs) are known to stimulate other angiogenic proteins including vascular endothelial growth factors (VEGFs). See, e.g., Seghezzi, et al., J. Cell. Biol., 141(7):1659-73 (1998); and Deroanne, et al., Cancer Res., 57(24):5590-97 (1997). Similarly, certain insulin-like growth factors (IGFs) and angiopoietins (Angs) have been shown to be capable of promoting and/or stimulating expression and/or activity of VEGF. See, e.g., Goad, et al, Endocrinology, 137(6):2262-68 (1996); Warren, et al., J. Bio. Chem., 271(46):29483-88 (1996); Punglia, et al, Diabetes, 46(10):1619-26 (1997); and Asahara, et al., Circ. Res., 83(3):233-40 (1998). Similarly, hepatocyte growth factor has been shown to increase expression of VEGF in human endothelial cells. See e.g. Wojta et al., Lab Invest. 79: 427-438, 1999. The nucleotide sequences of genes encoding these and other proteins, and the corresponding amino acid sequences are likewise known in the art (see, e.g., the GENBANK sequence database).

Fibroblast growth factors are a family of peptides that are typically potent regulators of cell proliferation, differentiation and function. At least nine members of the FGF family, FGF-1 through FGF-9, have been identified, and there are likely more to be found. FGF-1 and FGF-2, also known as acidic FGF (aFGF) and basic

FGF (bFGF), respectively, have been shown to be chemotactic and mitogenic for endothelial cells *in vitro*, inducing production of factors involved in the breakdown of the basement membrane and the migration of capillary endothelial cells into collagen matrices to form capillary-like tubes. See, e.g., Galzie, et al. Biochem Cell Biol, 75:669-685 (1997) and Gospodarowicz, et al. Endocr Rev, 8:95-114 (1987). The angiogenic activity of these and other members of the FGF family have been demonstrated *in vivo* and/or *in vitro*. See, for example, Klagsburn, M. Prog Growth Factor Res 1:207-235 (1989); Thompson, et al., PNAS, 86:7928-7932 (1989); Yanagisawa-Miwa, et al., Science, 257:1401-1403, (1992); Harada, et al. J. Clin. Invest., 94:623-630, (1994) and Unger, et al., Am. J. Physiol., 266:H1588-H1595, (1994) and have been successfully used for *in vivo* gene therapy applications as described by Hammond et al., supra. For additional details on the FGF family see, e.g., Burgess, Ann. N.Y. Acad. Sci. 638: 89-97, 1991; Burgess et al. Annu. Rev. Biochem 58:575-606, 1989; Muhlhauser et al. Hum. Gene Therapy 6:1457-1465, 1995; Zhan et al., Mol. Cell. Biol., 8:3487, 1988; Seddon et al. Ann. N.Y. Acad. Sci. 638:98-108, 1991; and, concerning human hst/KS3 (i.e. FGF-4), Taira et al. Proc. Natl. Acad. Sci. USA 84:2980-2984, 1987.

The vascular endothelial growth factor (VEGF) family of proteins includes, but is not limited to, members of the VEGF-A sub-family (e.g. VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206), the VEGF-B sub-family (e.g. VEGF-167 and VEGF-186), the VEGF-C and VEGF-D sub-families, as well as other VEGF-related proteins (VRPs), such as the poxvirus ORF-1 and ORF-2 proteins (sometimes referred to as members of a VEGF-E sub-family) and derivatives thereof. VEGFs (including VRPs) are typically mitogenic for cells (e.g. endothelial cells) and variously induce migration of endothelial cells and increase vascular permeability. See, for example, Eriksson and Alitalo, Vascular Growth Factors and Angiogenesis, Lena Claesson-Welsh (ed.), Springer (pub.) New York (1999) pp 41-57; and Ferrara, N., id. at pp 1-30, as well as Tischer et al. J. Biol. Chem. 206:11947-11954, 1991, and references therein; Muhlhauser et al., Cir. Res. 77:1077-1086, 1995; and Neufeld, *et al.*, WO 98/10071 published 12 Mar 1998. A number of modified or variant VEGF proteins have likewise been described and shown to be angiogenic. By way of

illustration, muteins of the VEGF-145 protein have been described by Neufeld, et al, WO 98/10071, published 12 Mar 1998; truncated VEGF proteins (including VRPs) have been described by Bohlen, et al., WO 98/07801, published 5 Nov 1998; and modified VEGFs including VEGF-138, VEGF-162, and VEGF-182, have been described by Baird, et al., WO 99/40197, published 12 Aug 1999, each of which is hereby incorporated by reference in its entirety.

In addition to chemical stimulation of angiogenesis, environmental conditions, such as low oxygen levels, or "hypoxia", can trigger and/or enhance angiogenesis. Certain proteins that are induced during hypoxia mediate an angiogenic response, such as hypoxia-inducible factors (Hifs). Hifs are themselves known to be capable of stimulating or potentiating other angiogenic proteins such as VEGFs. Such trans-activation or potentiation of one protein upon another is often mediated at the transcriptional level. For example, a first protein may stimulate or potentiate a second protein by directly or indirectly promoting expression of the gene encoding the second protein. Such proteins that promote angiogenesis by enhancing expression of a second angiogenic protein (e.g. by promoting transcription and/or by promoting or maintaining the stability of post-transcriptional products such as mRNA or protein) are referred to as angiogenic polypeptide regulators. For example, in the case of directly promoting expression, the first protein may activate a known or designed promoter of the second gene. In a second example, an angiogenic polypeptide regulator can modulate the stability of the second angiogenic protein via stabilization of the mRNA or protein. For example, these regulators can act on destabilizing elements in the 5' or 3' untranslated regions of the angiogenic transgene mRNA. A number of these stabilizing factors have been identified (see, e.g., Levy et al., J. Biol. Chem. 273: 6417-6423, 1998; Levy et al., J. Biol. Chem. 271: 25492-25497, 1996). These angiogenic polypeptide regulators of angiogenic genes are thus, inherently, angiogenic proteins; and genes encoding such angiogenic polypeptide regulators are themselves angiogenic genes. Since "rules" of promoter binding have been developed, it is also possible to design synthetic versions of such angiogenic polypeptide regulators, an exemplary class of which are the zinc finger proteins. (See, e.g., Rhodes and Klug, Scientific American, February 1993, pp 56-65; Choo and

Klug, PNAS, 91(23):11163-7 (1994); Rebar and Pabo, Science, 263(5147):671-3 (1994); Choo, et al., J Mol Biol, 273(3):525-32 (1997); Pomerantz et al., Science 267:93-96 (1995); and Liu, et al., PNAS, 94:5525-5530 (1997)). Angiogenic zinc finger proteins (AZFPs), designed to promote expression of other angiogenic genes, are thus, additional examples of angiogenic proteins and the genes encoding the AZFPs are thus additional examples of angiogenic genes that can be employed in the context of the present invention.

Insulin-like growth factors (IGFs) have likewise been shown to be angiogenic. By way of illustration, IGF-1 has been implicated as a stimulator of VEGF gene expression and is a known angiogenic protein (see, e.g., Punglia, et al., Diabetes, 46(10):1619-26 Oct 1997; Grant, et al., Diabetologia, 36(4):282-91 Apr 1993; Grant, et al., Ann. N Y Acad. Sci., 692:230-42 Aug 27 1993; Kluge, et al., Cardiovasc. Res., 29(3):407-15 Mar 1995; Necosia, et al., Am. J. Pathol., 145(5):1023-9 Nov 1994; and Delafontaine, P., Cardiovasc. Res., 30(6):825-34 Dec 1995).

Another example of angiogenic proteins or peptides is the family of angiopoietins. This family includes, for example, Ang1, Ang2 and Ang3 and derivatives thereof. Many angiopoietins are known to bind the TIE receptor family of receptor tyrosine kinases, which play an important role in angiogenesis. Studies of the angiopoietins have demonstrated their angiogenic activities, some of which are complementary to the angiogenic activity of other angiogenic proteins, such as, VEGFs. See, e.g., Thurston, et al., Science 286:2511-2514 (1999) and Davis and Yancopoulos, Vascular Growth Factors and Angiogenesis, Lena Claesson-Welsh, ed. Springer, publ. New York 1999, pp 173-185.

Additional examples of angiogenic proteins and peptides include hepatocyte growth factor (HGF), also known as scatter factor; placental growth factor (PlGF); transforming growth factor-beta (TGF-beta); platelet-derived growth factor (PDGF); epidermal growth factor (EGF); angiogenins; prostaglandin E1 and E2; endothelial cell stimulating angiogenesis factor; pleiotrophin; midkine; HIV tat protein; inducible nitric oxide synthase (iNOS) and its constitutive counterpart, cNOS, (regulatory factors induced by inflammation)AZFPs and derivatives thereof. Like the previously

discussed Hifs and AZFPs, these angiogenic proteins and peptides primarily act as regulators of angiogenesis by, for example, enhancing expression, stability or function of other angiogenic proteins or peptides. Thus, for example, studies have shown that Hif-1, a DNA-binding protein, is involved in the activation of VEGF transcription, as well as expression of the type II nitric oxide synthase (iNOS) gene, in response to hypoxia or ischemia. (See, e.g., Forsythe, et al., Mol. Cell Biol. 16(9):4604-4613 Sep. 1996; Palmer, et al., Am. J. Physiol. 274(2 Pt 1):L212-L219 Feb. 1998; Semenza, et al., Kidney Int. 51(2):553-555 Feb. 1997.) As discussed above, with respect to angiogenic zinc-finger proteins (or AZFPs), these regulatory proteins can be designed to specifically bind to sequences upstream of the coding regions of angiogenic genes and can be used to induce the expression of such genes. For rules describing the design of such zinc finger proteins see, e.g., Rhodes and Klug, Scientific American, February 1993, pp 56-65; Choo and Klug, PNAS, 91(23):11163-7 (1994); Rebar and Pabo, Science, 263(5147):671-3 (1994); Choo, *et al.*, J Mol Biol, 273(3):525-32 (1997); Pomerantz *et al.*, Science 267:93-96 (1995); and Liu, *et al.*, PNAS, 94:5525-5530 (1997).

Transgenes encoding analogs or derivatives of an angiogenic protein or polypeptide may be employed in the compositions of the present invention and are likewise contemplated herein. As is well known to those of skill in the art, useful analogs or derivatives generally have substantial sequence similarity (at the amino acid level) in regions or domains of the protein associated with the angiogenic activity. Additionally, such analogous or derivative proteins or peptides are functional equivalents retaining, to some extent, one or more activities of the related angiogenic protein or polypeptide. By "functional equivalent", it is meant that the analog has an activity which can be substituted for one or more activities of a particular angiogenic protein or polypeptide. Preferred functional equivalents retain most or more preferably all of the relevant activities of a particular angiogenic protein or polypeptide, however, the functional equivalent may have an activity that, when measured quantitatively, is stronger or weaker than the related angiogenic protein or peptide, as measured, for example, in functional assays, such as those disclosed herein. Preferred functional equivalents have activities that are within 1% to 10,000%

of the activity of the related angiogenic protein or polypeptide, more preferably at least 10%, and still more preferably at least 50%. The ability of a derivative to retain some activity can be measured using techniques known to those of skill in the art and/or described herein (See, e.g., the Examples provided below).

Specific types of derivatives or analogs include amino acid alterations such as deletions, substitutions, additions, and amino acid modifications. A "deletion" refers to the absence of one or more amino acid residue(s) in the related polypeptide. An "addition" refers to the presence of one or more amino acid residue(s) in the related polypeptide. Additions and deletions to a polypeptide may be at the amino terminus, the carboxy terminus, and/or internally. Amino acid "modification" refers to the alteration of a naturally occurring amino acid to produce a non-naturally occurring amino acid. A "substitution" refers to the replacement of one or more amino acid residue(s) by another amino acid residue(s) in the polypeptide. Derivatives can contain different combinations of alterations including more than one alteration and different types of alterations. Derivatives also include modifications occurring during or after translation, for example, phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand (see, e.g., Ferguson et al., Annu. Rev. Biochem., 57:285-320 (1988)).

While the effect of an amino acid change on the activity of an angiogenic protein or polypeptide varies depending upon factors such as phosphorylation, glycosylation, intra-chain linkages, tertiary structure, and the role of the amino acid in the active site or a possible allosteric site, it is generally preferred that the substituted amino acid is structurally and/or chemically similar to the amino acid being replaced. Thus, the following groups contain amino acids which tend to be relatively interchangeable: the basic amino acids lysine, arginine, and histidine; the acidic amino acids aspartic and glutamic acids; the neutral polar amino acids serine, threonine, cysteine, glutamine, asparagine and, to a lesser extent, methionine; the nonpolar aliphatic amino acids glycine, alanine, valine, isoleucine, and leucine (however, because of size, glycine and alanine are more closely related and valine, isoleucine and leucine are more closely related); and the aromatic amino acids phenylalanine, tryptophan, and tyrosine. In addition, although classified in different

categories, alanine, glycine, and serine tend to be interchangeable to some extent, and cysteine additionally fits into this group, or may be classified with the polar neutral amino acids.

Preferred derivatives have one or more amino acid alteration(s) which do not significantly affect the activity of the related angiogenic protein or polypeptide. In regions of the angiogenic protein or polypeptide not necessary for angiogenic activity, amino acids may be deleted, added or substituted with less risk of affecting activity. In regions required for angiogenic activity, amino acid alterations are less preferred as there is a greater risk of affecting angiogenic activity. Such alterations should preferably be conservative alterations. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent.

Conserved regions tend to be more important for protein activity than non-conserved regions. Standard procedures can be used to determine the conserved and non-conserved regions important for angiogenic activity using comparisons among related angiogenic proteins; as well as *in vitro* mutagenesis techniques to assess effects on angiogenic activity, as generally known to those of skill in the art.

Regulatory Sequences

Preferably, the transgenes employed in the present invention are operably linked to one or more promoters that direct transcription of the transgenes in a mammalian cell, such as a cell in the heart or in the skeletal muscle. Presently preferred promoters include generally constitutive promoters such as a cytomegalovirus immediate-early enhancer/promoter (herein referred to as "CMV promoter"), Rous sarcoma virus promoter (herein referred to as "RSV promoter"), Simian Virus 40 (herein referred to as "SV40 promoter") or human elongation factor-1 alpha/HTLV enhancer (herein referred to as "Hef-1 alpha / HTLV"). Other promoter systems include inducible systems (e.g., tetracycline-inducible, ecdysone and others). Alternatively, a tissue-specific promoter, such as a cardiac-specific promoter (e.g., a cardiomyocyte-specific promoter) may be employed. The promoter

is responsible for driving transcription of the angiogenic gene(s) and the selection of the appropriate promoter is based on published data as well as empirical evidence as illustrated herein. Many promoters and promoter systems are commercially available through vendors such as Stratagene (La Jolla, CA), Invitrogen (Carlsbad, CA), Promega (Madison, WI), Invivogen (San Diego, CA), and others. Preferably, the transgenes are also operably linked to a polyadenylation signal and may likewise be linked to enhancers or similar regulatory sequences.

In presently preferred embodiments, the two transgenes are contained within a single gene delivery vector with expression of each transgene resulting in production of a separate angiogenic protein or peptide. Preferably, each transgene is associated with its own promoter sequence (as well as any other regulatory sequences desired or needed). By way of illustration, the two transgenes can be provided in a tandem (head-to-tail) orientation within the gene delivery vector, that is, with a first promoter followed by the coding sequence of the first angiogenic transgene, followed by a second promoter which is followed by the coding sequence of the second angiogenic transgene. In this configuration, the operons (i.e., each transgene and its associated regulatory sequences) may be linked to one another (i.e., $P_1 \rightarrow A_1 \rightarrow P_2 \rightarrow A_2$, where each P represents a promoter sequence and each A represents a transgene encoding an angiogenic protein or peptide and no sequences intervene between the two operons) or the operons may be adjacent to one another (i.e., $P_1 \rightarrow A_1 \rightarrow X \rightarrow P_2 \rightarrow A_2$, wherein each P and A are as above, and X represents an intervening untranslated nucleotide sequence, typically referred to as a "spacer"). Alternatively to the head-to-tail orientation, the two transgenes may be oriented head-to-head (i.e., $A_1 \leftarrow P_1\text{-}X\text{-}P_2 \rightarrow A_2$). In yet another alternative, the operons may be configured in a tail-to-tail orientation (i.e., $P_1 \rightarrow A_1 \rightarrow\text{-}X\text{-}\leftarrow A_2 \leftarrow P_2$). In this orientation, appropriate intervening nucleotide sequences may be incorporated to reduce or prevent read-through during transcription.

The promoters employed to drive expression of the transgenes may be the same or different from one another. The promoters may include, for example and without limitation, CMV, RSV, Hef-1 alpha, or other constitutive promoters,

inducible promoters, such as tetracycline-regulatable or other regulatable promoters or tissue-specific promoters, e.g. cardiomyocyte-specific promoters. Numerous arrangements of promoters and transgenes, some of which are illustrated in detail below, may be used in accordance herewith.

In other embodiments, a single promoter may be employed to effect transcription of both transgenes wherein a single promoter may be employed to yield two separate proteins or peptides by providing each transgene with its own stop codon such that the single promoter effects transcription of each angiogenic transgene individually. Use of a single promoter to drive two transgenes may be employed but typically the transgene furthest downstream from the promoter is expressed less efficiently than its upstream neighbor. In a second exemplary illustration, the single promoter may be used to yield two separate angiogenic proteins or peptides by providing an IRES (internal ribosome entry site) sequence between the two transgenes, resulting in translation of the single transcription product into two translation products. Transcription of constructed bi-cistronic transgenes has been shown to be enhanced by inserting an IRES sequence, most optimally between 100 and 500 nucleotides after the termination codon of the first transgene (see e.g. Attal J., et al., Genet. Anal. 15(3-5): 161-5, 1999). Those of skill in the art will appreciate that various promoter/transgene configurations may be employed in accordance herewith.

A single promoter may also be employed to yield a single translation product. For example, the two angiogenic transgenes can be effectively fused, such that, together with the promoter and other regulatory sequences, they form a single operon yielding a transcriptionally fused product which is also translationally fused, i.e., fused into a single gene, translation of which results in a single bifunctional peptide. The coding regions of such a bifunctional peptide may include a polynucleotide spacer sequence between the domains to separate the domains in the mature peptide. Typically, such a spacer has a length of no more than about 0.5 kilobases, more preferably about 0.1 kb (and contains no stop codons). In an exemplary embodiment, the spacer is configured to encode a short, flexible polypeptide sequence (or

"flexon"), such as a polyglycine sequence for example, to facilitate optimal, independent folding and activity of the two angiogenic peptides on either side thereof.

As discussed further below, the angiogenic transgenes can also be provided in separate vectors, which vectors may be the same or different from one another. In this embodiment, because the transgenes are contained in different gene delivery vehicles, each transgene is necessarily associated with its own promoter and other regulatory sequences. Alternative configurations will be readily appreciated by those of skill in the art. For example, one vector may comprise a first transgene, with associated promoter, polyadenylation sequence and the like, and a second vector may comprise second and third transgenes that are in any of various configurations as discussed above.

Other post-transcription and/or post-translation signaling sequences may likewise be included in the transgenes in accordance with the present invention. For example, while a number of angiogenic proteins include a natural secretory signal that directs secretion of the protein into the extracellular space, some do not include such a signal. Thus, in preferred embodiments, where no native secretory signal is present, a nucleotide sequence encoding a secretory signal sequence is operably linked to the transgene encoding that angiogenic protein. By way of illustration, examples of angiogenic proteins/peptides that have a native secretory signal include FGF-4, FGF-5, FGF-6 and many, if not most, VEGFs (including, without limitation, members of the VEGF-A, VEGF-B and VEGF-C sub-families). Most angiogenic proteins or peptides containing a native secretory signal are readily secreted and diffusible after secretion. Thus, these angiogenic proteins, when expressed, can readily access the cardiac interstitium and induce angiogenesis. Blood vessels that can develop during angiogenesis include capillaries (which are the smallest caliber blood vessels having a diameter of about 8 microns), as well as larger caliber blood vessels that have a diameter of at least about 10 microns. Thus, in one aspect, angiogenic activity of a protein or peptide can be determined by measuring blood flow and/or vascularization and/or an increase in the function of a treated tissue, e.g., an ischemic tissue, using procedures known in the art or described herein. For example, blood flow can be assessed by contrast echocardiography, or by quantification of capillary number or

density, which, in an animal, can be quantitated visually or by microscopic analysis of the tissue site (see, e.g., Hammond, et al., WO 96/26742, published 6 Sep. 1996; Helmer, et al., Circulation, 94(9):2260-67 (1996); Giordano, et al., Nat. Med., 2(5):534-9 (1996), and the Examples below).

With other angiogenic proteins such as aFGF (FGF-1) and bFGF (FGF-2) that lack a native secretory signal sequence, fusion proteins having secretory signal sequences can be recombinantly produced using standard recombinant DNA methodology familiar to those of skill in the art. It is believed that both aFGF and bFGF are naturally secreted to some degree; however, inclusion of an additional secretion signal sequence can be used to enhance secretion of the protein. The secretory signal sequence would be placed at any position suitable to allow secretion of the angiogenic factor (typically at the N-terminus of the desired protein). For example, a polynucleotide containing a suitable signal sequence can be fused 5' to the first codon of the selected angiogenic protein gene. Suitable secretory signal sequences include, for example, signal sequences of the FGF-4, FGF-5, FGF-6 genes, as well as signal sequences from a different secreted protein such as, for example, IL-1-beta or other signal sequence, especially one derived from a protein that is normally secreted from cardiac myocytes. Construction of transgenes encoding angiogenic proteins or peptides fused to a signal sequence may be accomplished in a variety of ways, known to those of skill in the art. (See, e.g., Bohlen, et al., WO 98/49300, published 5 Nov 1998.)

Angiogenic genes (i.e., genes encoding proteins capable of promoting or enhancing angiogenesis) can also provide additional functions that can be useful for treating the cardiovascular patient, such as, for example, improvement of cardiac cell function. By way of illustration, FGFs can provide cardiac enhancing and/or "ischemic protectant effects" that may be independent of their capability to promote angiogenesis. Thus, angiogenic genes can be used to enhance cardiac function by mechanisms that are additional to or in place of the promotion of angiogenesis per se. As an additional example, IGFs, which can promote angiogenesis, can also enhance muscle cell function (see e.g. Musaro et al., Nature 400: 581-585, 1999). Other

proteins which enhance muscle cell or other cardiac cell function can likewise be employed in accordance with the present invention.

While any combination of angiogenic transgenes may generally be employed in the present compositions, the transgenes are selected preferably to encode angiogenic proteins or peptides that are different from one another and, more preferably are from different angiogenic gene families. Selecting the angiogenic transgenes to be from different families may, for example, enhance the overall angiogenic potential of the composition as compared to an individual angiogenic transgene. It can also improve overall efficacy ratios since individual patients may respond to one angiogenic protein more strongly than another. In addition, providing multiple angiogenic factors may promote a more robust angiogenic response.

By way of illustration, each angiogenic protein or peptide may be selected to affect different angiogenic pathways or to affect the same pathway at different points. As is well known in the art, a number of *in vitro* and *in vivo* assays may be employed to compare the angiogenic activity of different proteins/peptides and combinations thereof, some of which are exemplified by way of illustration herein. Similarly, individual patients may be more responsive to one angiogenic protein than to another. Thus, providing two different proteins can also reduce the incidence of "non-responders" to a particular therapy.

In preferred embodiments, the angiogenic activity of the combined transgenes is equal to or, more preferably greater, quantitatively and/or qualitatively, than that of each individual transgene. Quantitatively, for example, the activity of the combined transgenes is preferably greater than the sum of the individual activities, more preferably at least four times the individual activities and most preferably at least 10 times the individual activities. The combination may also be qualitatively different from each transgene alone in that the combination is capable of eliciting biological effects that are different and potentially far superior to the effects seen with either transgene alone. In that regard, transgenes selected from different angiogenic families or types are generally preferred. By way of illustration, in preferred embodiments exemplified herein, one transgene encodes a member of the FGF family of angiogenic

proteins and another transgene encodes a member of the VEGF family of angiogenic proteins. Other combinations of transgenes and/or vectors will be apparent to those of skill in the art based on the teachings and illustrations of the present invention.

For treating humans, transgenes encoding angiogenic proteins of human origin are preferred although angiogenic proteins of other mammalian origin, that exhibit cross-species activity (i.e. having angiogenic activity in humans), can also be used.

Isolation/Construction of Transgenes

There are various methods known to those of skill in the art for isolating and/or producing nucleic acid molecules for the production of compositions in accordance with the present invention. For example, the desired angiogenic gene may be isolated from a DNA library, such as a human cDNA library, or it may be synthesized (e.g. By RT-PCR from total tissue RNA). Construction of exemplary angiogenic genes is illustrated in the Examples below, although other techniques can also be used as will be appreciated by those of skill in the art.

Where the angiogenic nucleic acid molecule is a derivative of a known angiogenic gene, it may be constructed, for example, from the existing, related angiogenic gene. The related gene may, however, be modified, for example, by site-directed mutagenesis or by other methods known to those of skill in the art and/or described herein. Thus, standard recombinant techniques for mutagenesis such as *in vitro* site-directed mutagenesis (Hutchinson *et al.*, J. Biol. Chem. 253:6551, (1978)), Sambrook *et al.*, Chapter 15, *supra*, use of TAB® linkers (Pharmacia), and PCR-directed mutagenesis can be used to create desired derivatives. Alternatively, nucleic acid molecules encoding angiogenic proteins or peptides may be synthesized, for example, by the chemical methods or by using an automated DNA synthesizer.

Gene Delivery Vectors

Vectors useful in the present invention include viral vectors, lipid-based vectors (e.g. liposomes) and other vectors that are capable of delivering DNA to cells *in vivo*. Presently preferred are viral vectors, particularly replication-deficient viral

vectors including, for example, replication-deficient adenovirus and adeno-associated virus vectors. For ease of production and use in the present invention, replication-deficient adenovirus vectors are exemplified herein. In contrast to some other viral delivery systems, adenovirus generally does not require host cell replication for gene expression because integration is not normally a component of the adenoviral life cycle. Thus, adenovirus can infect non-dividing cells making it well suited for expressing recombinant genes in the myocardium given the generally nonreplicative nature of cardiac myocytes.

A variety of other vectors, both viral and non-viral, can likewise readily be employed to deliver angiogenic transgenes in accordance with the present invention. Preferably, vectors suitable or modifiable for *in vivo* gene delivery will be selected. With respect to viral vectors, adeno-associated virus (AAV), lentivirus (e.g. based on HIV, feline immunodeficiency virus), herpes virus vaccinia virus, various RNA viruses and bovine papilloma virus are exemplary. By way of illustration, AAV vectors useful in the gene therapy methods and compositions of the present invention are preferably replication-deficient in humans, for example, due to deletion of the *rep* and/or *cap* genes, essential to AAV replication, and the transgenes (including associated promoters and other regulatory sequences) inserted therein are preferably flanked by AAV inverted terminal repeat (ITR) sequences. The resulting recombinant AAV vector is then replicated in a packaging cell line supplying the missing AAV functions (i.e., the rep and/or cap genes) in trans. References describing these and other gene delivery vectors are known in the art, a number of which are cited herein.

As described above and in the scientific literature, a number of retrovirus-derived systems have also been developed to be used in *in vivo* gene delivery. By way of illustration, the lentivirus genus of retroviruses (for example, human immunodeficiency virus, feline immunodeficiency virus and the like) can be modified so that they are able to transduce cells that are typically non-dividing (see, e.g., Naldini et al., Science 272:263-267, 1996; Miyoshi et al., J. Virol. 72:8150-8157, 1998; and Buchschacher et al., Blood 15:2499-2504 2000; see also Verma et al., U.S. Patent 6,013,516 (January 11, 2000)). While HIV-based lentiviral vector systems have received some degree of focus in this regard, other lentiviral systems have

recently been developed, such as feline immunodeficiency virus-based lentivirus vector systems, that offer potential advantages over the HIV-based systems (see e.g. Poeschla et al., Nat. Med. 4:354-357, 1998; see also the review by Romano et al., Stem Cells 18:19-39, 2000 and references reviewed therein).

In addition to viral vectors, non-viral vectors that may be employed as a gene delivery means are likewise known and continue to be developed. For example, non-viral protein-based delivery platforms, such as macromolecular complexes comprising a DNA binding protein and a carrier or moiety capable of mediating gene delivery, as well as lipid-based vectors (such as liposomes, micelles, lipid-containing emulsions and others) have been described in the art (see e.g. Romano et al., Stem Cells 18:19-39, 2000 and references reviewed therein). Improvements in lipid-mediated *in vivo* gene delivery have been facilitated by the development of new cationic formulations and vector delivery co-factors (see e.g. Kollen et al., Hum. Gene Ther. 10:615-22, 1999; Roy et al., Nat. Med. 5:387-391; Fajac et al., Hum. Gene Ther. 10:395-406, 1999; Ochiya et al., Nat. Med. 5:707-710, 1999). Additionally, the development of systems which combine components of viral and non-viral mediated gene delivery systems have been described and may be employed herein (see e.g. Di Nicola et al., Hum. Gene Ther. 10:1875-1884, 1999).

As mentioned above, the compositions and methods of the present invention can also employ multiple vectors to deliver the angiogenic transgenes. Thus, one of the transgenes can be provided in one vector and the second transgene provided in the same or a different vector. Such vectors can be delivered to the patient concomitantly or in series. In preferred embodiments the vectors are selected to be different from one another. Preferably, the vectors are selected, in conjunction with the transgenes, to facilitate the gene therapy. By way of illustration, in an exemplary embodiment of a dual vector system, one transgene is provided in an adenovirus (Ad) vector and a second transgene is provided in an adeno-associated virus (AAV) vector. In addition to transfection efficiency, the choice of vector may be influenced by the desired longevity of transgene expression. For example, a transgene that can bring about long-term effects without requiring long-term expression (e.g., by initiating or facilitating the process of angiogenesis which results in an increase in tissue

vascularization) may be introduced using an adenovirus (or other vector that does not normally integrate into host DNA) which might be used prior to or in combination with the introduction of an AAV vector carrying a transgene for which longer-term expression is desired.

Other vector types may likewise be combined to deliver two angiogenic genes in accordance with the methods and compositions herein. Each different vector type can further be employed to carry more than one transgene. Thus, for example, one or multiple transgenes encoding the same or different angiogenic proteins or peptides can be contained within one vector type and one or multiple additional angiogenic transgenes can be contained within a second vector type. Most preferably, the compositions comprise at least two different angiogenic transgenes in a single vector or comprise at least two angiogenic transgenes, whether the same or different from one another, in at least two different vectors. Numerous configurations of transgenes and vectors, in accordance herewith, will be apparent to those of skill in the art and are likewise contemplated herein.

Recombinant viral vectors comprise heterologous in non-viral genes or sequences. Since many viral vectors exhibit size-constraints associated with packaging, and since replication-deficient viral vectors are generally preferred for *in vivo* delivery, the heterologous genes or sequences are typically introduced by replacing one or more portions of the viral genome. Such viruses may become replication-deficient as a result of the deletions, thereby requiring the deleted function(s) to be provided *in trans* during viral replication and encapsidation (by using, e.g., a helper virus or a packaging cell line carrying genes necessary for replication and/or encapsidation) (see, e.g., the references and illustrations of viral vectors herein). Modified viral vectors in which a polynucleotide to be delivered is carried on the outside of the viral particle have also been described (see, e.g., Curiel, DT, et al. PNAS 88:8850-8854, 1991).

In a further aspect of the present invention, where the compositions are employed in gene therapy methods, the efficiency of gene delivery using a vector such as a viral vector (e.g. adenovirus or adeno-associated virus) is enhanced by

delivering the vector into a blood vessel or into a tissue that is co-infused or pre-infused with a vasoactive agent, for example histamine, a histamine agonist, a vascular endothelial growth factor (VEGF) protein or a nitric oxide donor, such as sodium nitroprusside. The use of such vasoactive agents has been described and illustrated by e.g., Hammond, et al., in USSN 09/021,773, filed 11 February 1998; WO99/40945 published 19 August 1999; and USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by In Vivo Gene Delivery", each of which hereby incorporated by reference in their entirety. Most preferably the vasoactive agent is infused into the blood vessel or tissue coincidently with or within several minutes prior to introduction of the vector composition. Vasoactive agent, as used herein, refers to a natural or synthetic substance that induces increased vascular permeability and/or enhances transfer of macromolecules such as gene delivery vectors from blood vessels, e.g. across capillary endothelia. By augmenting vascular permeability to macromolecules or otherwise facilitating the transfer of macromolecules into the capillary bed perfused by an artery (or served by a vein), vasoactive agents can enhance delivery of these vectors to the targeted sites and thus effectively enhance overall expression of the transgene in the target tissue. By way of illustration, histamine has been used as a vasoactive agent and was found to substantially enhance delivery of a vector to an infused site such as the myocardium. See, e.g., Hammond, et al., WO 99/40945, published 19 Aug 1999. Histamine derivatives and agonists, such as related compounds that interact with histamine receptors, which can be employed include, for example, 2-methylhistamine, 2-pyridylethylamine, betahistine, and 2 thiazolylethylamine. These and additional histamine agonists are described, for example, in Garrison JC., Goodman and Gilman's The Pharmacological Basis of Therapeutics (8th Ed: Gilman AG, Rall TW, Nies AS, Taylor P, eds) Pergamon Press, 1990, pp 575-582 and in other pharmacological treatises. In addition or alternatively to histamine or histamine agonists, vascular endothelial growth factors (VEGFs), VEGF agonists (as described herein and in the cited references) or a nitric oxide donor (e.g., sodium nitroprusside) can be used to induce increased vascular permeability and can therefore be used as a vasoactive agent to enhance gene delivery in the context of the compositions and methods described herein. As with histamine, the vasoactive agent is preferably

infused into a blood vessel supplying the target site over several minutes prior to infusion of vector.

Limiting expression of the angiogenic transgene to the heart, or to particular cell types within the heart (e.g. cardiac myocytes), or to other target tissues, such as those in the peripheral vasculature, can provide certain advantages as discussed herein.

The present invention contemplates the use of targeting not only by delivery of vector into a coronary artery or other tissue-specific conduit, for example, but also by use of targeted vector constructs having features that tend to target gene delivery and/or gene expression to particular host cells or host cell types (e.g. cardiac or other myocytes). Such targeted vector constructs would thus include targeted delivery vectors and/or targeted vectors, as described in more detail below and in the published art. Restricting delivery and/or expression can be beneficial as a means of further focusing the potential effects of gene therapy. The potential usefulness of further restricting delivery/expression depends in large part on the type of vector being used and the method and place of introduction of such vector. As described herein, delivery of viral vectors via intracoronary injection to the myocardium has been observed to provide, in itself, highly targeted gene delivery. However, other means of limiting delivery and/or expression can also be employed, in addition to or in place of the illustrated delivery methods, as described herein.

Targeted delivery vectors include, for example, vectors (such as viruses, non-viral protein-based vectors and lipid-based vectors) having surface components (such as a member of a ligand-receptor pair, the other half of which is found on a host cell to be targeted) or other features that mediate preferential binding and/or gene delivery to particular host cells or host cell types. As is known in the art, a number of vectors of both viral and non-viral origin have inherent properties facilitating such preferential binding and/or have been modified to effect preferential targeting (see, e.g., Douglas et al., Nat. Biotech. 14:1574-1578, 1996; Kasahara, N. et al. Science 266:1373-1376, 1994; Miller, N., et al., FASEB Journal 9: 190-199, 1995; Chonn, A., et al., Curr. Opin. in Biotech. 6: 698-708, 1995; Schofield, JP, et al., British Med.

Bull. 51: 56-71, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68: 145-159, 1994; Ledley, F.D., Hum. Gene Ther. 6: 1129-1144, 1995; Conary, J.T., et al., WO 95/34647 (21 December 1995); Overell, R.W., et al., WO 95/28494 (26 October 1995); and Truong, V.L. et al., WO 96/00295 (4 January 1996)).

As stated above and in the cited references, vectors can also comprise components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. Such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector by the cell; components that influence processing and/or localization of the vector and its nucleic acid within the cell after uptake (such as agents mediating intracellular processing and/or nuclear localization); and components that influence expression of the polynucleotide. Such components can also include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be modified to provide such functionalities (such as viruses which have been modified to include a cell binding or targeting protein on the exterior surface of their envelope or capsid). A detectable marker gene allows cells carrying the gene to be specifically detected (e.g., distinguished from cells which do not carry the marker gene). One example of such a detectable marker gene is the lacZ gene, encoding beta-galactosidase, which allows cells transfected with a vector carrying the lacZ gene to be detected by staining, as described below. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. A variety of such marker genes have been described, including bifunctional (i.e. positive/negative) markers (see, e.g., Lupton, S., WO 92/08796, published 29 May 1992; and Lupton, S., WO 94/28143, published 8 December 1994). Such marker genes can provide an added measure of control that

can be advantageous in gene therapy contexts. A large variety of such vectors are known in the art and are generally available (see, e.g., the various references cited above).

References describing adenovirus vectors and other viral vectors which could be used in the compositions and methods of the present invention, in addition to those cited above, include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) Virology, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F., et al., pp. 109128 in Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., FASEB Journal 9:190-199, 1995; Schreier, H, Pharmaceutica Acta Helvetiae 68:145-159, 1994; Schneider and French, Circulation 88:1937-1942, 1993; Curiel D.T., et al., Human Gene Therapy 3:147-154, 1992; Graham, F.L., et al., WO 95/00655 (5 January 1995); Falck-Pedersen, E.S., WO 95/16772 (22 June 1995); Denefle, P. et al., WO 95/23867 (8 September 1995); Haddada, H. et al., WO 94/26914 (24 November 1994); Perricaudet, M. et al., WO 95/02697 (26 January 1995); Zhang, W., et al., WO 95/25071 (12 October 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996).

Additional references describing AAV vectors which could be used in the compositions and methods of the present invention include the following: Carter, B., Handbook of Parvoviruses, vol. 1, pp. 169-228, 1990; Berns, Virology, pp. 1743-1764 (Raven Press 1990); Carter, B., Curr. Opin. Biotechnol., 3:533-539, 1992; Muzyczka, N., Current Topics in Microbiology and Immunology, 158:92-129, 1992; Flotte, T.R., et al., Am. J. Respir. Cell Mol. Biol. 7:349-356, 1992; Chatterjee et al., Ann. NY Acad. Sci., 770:79-90, 1995; Flotte, T.R., et al., WO 95/13365 (18 May 1995); Trempe, J.P., et al., WO 95/13392 (18 May 1995); Kotin, R., Human Gene Therapy, 5:793-801, 1994; Flotte, T.R., et al., Gene Therapy 2:357-362, 1995; Allen, J.M., WO 96/17947 (13 June 1996); and Du et al., Gene Therapy 3:254261, 1996; Kaplitt et al., Ann. Thorac. Surg. 62: 1669-1676, 1996; Samulski et al., J. Virol. 63: 3822-3828,

1989; Zolotukhin et al., Gene Therapy 6: 973-985, 1999; Atkinson et al., WO 99/11764 (11 March 1999).

References describing non-viral vectors which could be used in the composition and methods of the present invention include the following: Ledley, FD, Human Gene Therapy, 6:11 29-1144, 1995; Miller, N., et al., FASEB Journal, 9:190-199, 1995; Chonn, A., et al., Curr. Opin. in Biotech.. 6:698-708, 1995; Schofield, JP, et al., British Med. Bull., 51:56-71, 1995; Brigham, K. L., et al., J. Liposome Res., 3:31 49, 1993; Philip, R., et al., Mol. Cell Biol. 14: 2411-2418, 1994; Perales et al., Proc. Natl. Acad. Sci. U.S.A. 91: 4086-4090, 1994; Hanson et al., WO 95/25809 (28 September 1995); Gao et al., WO 96/22765 (1 August 1996); Brigham, K.L., WO 91/06309 (16 May 1991); Felgner, P.L., et al., WO 91/17424 (14 November 1991); Solodin et al., Biochemistry 34:13537-13544, 1995; WO 93/19768 (14 October 1993); Debs et al., WO 93125673; Felgner, P.L., et al., U.S. Patent 5,264,618 (November 23, 1993); Epand, R.M., et al., U.S. Patent 5,283,185 (February 1, 1994); Gebeyehu et al., U.S. Patent 5,334,761 (August 2, 1994); Felgner, P.L., et al., U.S. Patent 5,459,127 (October 17, 1995); Overell, R.W., et al., WO 95/28494 (26 October 1995); Jessee, WO 95/02698 (26 January 1995); Haces and Ciccarone, WO 95/17373 (29 June 1995); Lin et al., WO 96/01840 (25 January 1996).

Construction of An Exemplary Recombinant Adenoviral Vector

In one aspect of the present invention, a recombinant replication-deficient adenoviral vector, comprising two transgenes, each encoding an angiogenic protein or peptide, is provided. In addition to other methods known to those of skill in the art, such a recombinant adenoviral vector may be constructed using the rescue recombination technique (as described for example in Graham, Virology 163:614-617, 1988).

Briefly, in an exemplary illustration, the transgenes of interest are cloned into a shuttle vector that contains a promoter (if not already linked to the transgene(s)), a polylinker and partial flanking adenoviral sequences from which E1A and E1B genes have been deleted. A shuttle vector, which encodes portions of the left end of the

human adenovirus 5 genome minus the early protein encoding ElA and ElB sequences that are essential for viral replication (see, e.g., Virology 163: 614-617, 1988) and analogous shuttle vectors, a number of which are commercially available, and plasmid ACCMVpLpA (J Biol Chem 267:25129-25134, 1992) (which contains polylinker, the CMV promoter and SV40 polyadenylation signal flanked by partial adenoviral sequences also lacking the ElA/ElB genes) can be exemplified. The use of plasmid pAC1 or ACCMVpLpA facilitates the cloning process.

The shuttle vector is then co-transfected into 293 cells (which contain Adenovirus E1 sequences capable of complimenting the missing Adenoviral genes) with a plasmid which contains the entire human adenoviral 5 genome with a length too large to be encapsidated. Co-transfection can be conducted, for example, by calcium phosphate precipitation or lipofection (Biotechniques 15:868-872, 1993) or other techniques known to those skilled in the art. Plasmid JM17 (pJM17) encodes the entire human adenovirus 5 genome plus portions of the vector pBR322 including the gene for ampicillin resistance (4.3 kb). Although JM17 encodes all of the adenoviral proteins necessary to make mature viral particles, the inclusion of the additional sequences (i.e., pBR322 sequences) renders it too large to be encapsidated (40 kb versus 36 kb for wild type). In a small subset of co-transfected cells, rescue recombination between the transgene-containing shuttle vector and the plasmid having the entire adenoviral 5 genome provides a recombinant genome that is deficient in the ElA/ElB sequences and that contains the transgenes of interest but secondarily loses the additional sequence, such as the pBR322 sequences, during recombination, thereby being small enough to be encapsidated.

After identification and purification of successful recombinants, viral stocks are propagated in 293 cells to titers typically ranging between 10^8 and 10^{13} viral particles. The adenoviral constructs are then purified to provide high titer, high purity stocks preferably with less than about one (1) replication competent adenovirus (RCA) particle per million, more preferably with fewer than 1 per 10^9 and most preferably with fewer than 1 per 10^{12}.

Therapeutic Applications

In the examples provided herein, the present method of *in vivo* transfer of at least two transgenes encoding angiogenic proteins or peptides is used to demonstrate that gene transfer of a recombinant adenovirus expressing at least two angiogenic proteins or peptides is effective in substantially reducing myocardial ischemia. As the data below shows, expression of the angiogenic transgenes resulted in increased blood flow and/or function in the target tissue (i.e., the heart), even at very low viral particle dosages.

As those of skill in the art will appreciate and as described elsewhere herein, various types of vectors can be employed to deliver the angiogenic transgenes, *in vivo*, according to the present invention. For example, adenovirus, adeno-associated virus and other viral vectors, as well as non-viral vectors such as lipid-based gene delivery systems and "naked DNA" vector systems can be used. In one example, provided herein, replication-deficient recombinant adenovirus vectors are exemplified, resulting in highly efficient gene transfer, *in vivo*, at substantially lower dosage levels than single-gene-containing vectors.

The compositions of the present invention (such as, for example, those employing a replication-deficient adenovirus) allow for highly efficient gene transfer *in vivo* without significant necrosis or inflammation. Based on these results, some of which are described in detail in the Examples below, it is seen that a sufficient degree of *in vivo* gene transfer to effect *in vivo* functional changes is achieved. The gene transfer of at least two transgenes encoding angiogenic proteins or peptides will improve blood flow and/or enhance muscle function in the treated tissue (e.g. the myocardium).

In one aspect, the compositions and methods of the present invention can be employed to treat dilated cardiomyopathy (DCM), a type of heart failure that is typically diagnosed by the finding of a dilated, hypocontractile left and/or right ventricle. DCM can occur in the absence of other characteristic forms of cardiac disease such as coronary occlusion or a history of myocardial infarction. DCM is

generally associated with poor ventricular function and symptoms of heart failure. In these patients, chamber dilation and wall thinning generally results in a high left ventricular wall tension. Many patients exhibit symptoms even under mild exertion or at rest, and are thus characterized as exhibiting moderately severe to severe, i.e. Class III or Class IV, heart failure, respectively (based on the New York Heart Association (NYHA) Functional Classification of cardiovascular disease). As noted above, many patients with coronary artery disease may progress to exhibiting dilated cardiomyopathy, often as a result of one or more heart attacks (myocardial infarctions).

A further application of the present invention is to prevent, or at least substantially alleviate, deleterious left ventricular remodeling (also referred to simply as remodeling), which refers to chamber remodeling after myocardial infarction that can cause and/or exacerbate heart failure. Even if ventricular remodeling has already initiated, it is still desirable to take steps to improve myocardial function, as this may still be effective to reduce ventricular remodeling and potentially prevent or lessen the severity of congestive heart failure (see, generally, Yang, et al., Chin. Med. J. 111(2):142-146 1998).

Similarly, promotion of angiogenesis can be useful, since the development of an improved microvascular bed can also be effective to offset ventricular dysfunction. Further, such angiogenic proteins or peptides can also have other effects that improve cardiac function, such as increasing blood flow (see, e.g., Giordano, et al., Nat. Med., 2(5):534-9,1996) as well as having protective effects after ischemic events or infarction (see e.g. Buerke et al., Proc. Natl. Acad. Sci. U.S.A., 92: 8031-8035, 1995; Li et al., J. Clin. Invest. 100: 1991-1998, 1997). Any observable or measurable reduction in an existing symptom of the heart failure may indicate alleviation of deleterious ventricular remodeling. For example, the patient may show less breathlessness and/or improved exercise tolerance. Methods of assessing improvement in heart function and reduction of symptoms are essentially analogous to those described above for DCM.

In treating angina, as may be associated with CAD, transfer of transgenes encoding angiogenic proteins or peptides can be conducted at any time, but preferably is performed relatively soon after the onset of severe angina. In treating most congestive heart failure, gene transfer of transgenes encoding angiogenic proteins or peptides can be conducted, for example, after diagnosis of heart failure or the likely development of heart failure. For treating events associated with a myocardial infarct, gene transfer can be performed at any time after the patient has suffered the infarct, preferably within 30 days, more preferably within 3-20 days after an infarct, most preferably within the first day following infarct.

Compositions of the present invention may conveniently be provided in the form of formulations suitable for administration to a patient, into the blood stream (e.g. by intra-arterial injection or as a bolus infusion into tissue such as the skeletal muscle). A suitable final administration format is best determined by a medical practitioner. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulations treatises, *e.g., Remington's Pharmaceuticals Sciences* by E.W. Martin. *See also* Wang, Y.J. and Hanson, M.A., "Parental Formulations of Proteins and Peptides: Stability and Stabilizers", *Journals of Parental Sciences and Technology,* Technical Report No. 10, Supp. 42:2S (1988). Vectors of the present invention are preferably formulated in solutions at or near neutral pH, for example, about pH 6.5 to about pH 8.5, more preferably from about pH 7 to 8, preferably with an excipient to bring the solution to near isotonicity, for example, 4.5% mannitol or 0.9% sodium chloride, pH buffered with art-known buffer solutions, such as sodium phosphate, that are generally regarded as safe, typically together with an accepted preservative such as metacresol 0.1% to 0.75%, more preferably from 0.15% to 0.4% metacresol. The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions. By way of illustration, an exemplary formulation comprises the vector(s) in a phosphate buffered saline (PBS) solution at pH7 with 2 mM magnesium chloride ($MgCl_2$) and 2% w/v sucrose. If desired, solutions of the

compositions also can be prepared to enhance shelf life and stability. The therapeutically useful compositions of the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be mixed to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water and/or a buffer to control pH or an additional solute to control tonicity.

A preferred administration of the compositions of the present invention is by injection into one or more localized sites (e.g., one or both coronary arteries, in the case of heart diseases) using a suitable catheter or other *in vivo* delivery device. Preferably, the vector composition is administered into a vessel supplying blood to the region of tissue in need of therapy, i.e., an anterograde administration. However, it will be appreciated by those of skill in the art that administration into a vessel receiving blood from the region of tissue in need of therapy, i.e. retrograde administration via a vein or into the coronary sinus that receives blood from the myocardium, may likewise be employed. See, for example, Boekstegers, et al., Journal of the American College of Cardiology, 31(7):1525-1533 (1998).

A variety of catheters and delivery routes can be used to achieve intracoronary delivery, as is known in the art (see, e.g., the references cited above, including: Topol, EJ (ed.), The Textbook of Interventional Cardiology, 2nd Ed. (W.B. Saunders Co. 1994); Rutherford, RB, Vascular Surgery, 3rd Ed. (W.B. Saunders Co. 1989); Wyngaarden JB et al. (eds.), The Cecil Textbook of Medicine, 19th Ed. (W.B. Saunders, 1992); and Sabiston, D, The Textbook of Surgery, 14th Ed. (W.B. Saunders Co. 1991)). Direct intracoronary (or graft vessel) injection can be performed using standard percutaneous catheter based methods under fluoroscopic guidance. Any variety of coronary catheter, or a Stack perfusion catheter, for example, can be used in the present invention. For example, a variety of general purpose catheters, as well as modified catheters, suitable for use in the present invention are available from commercial suppliers such as Advanced Cardiovascular Systems (ACS), Target Therapeutics, Boston Scientific and Cordis. Also, where delivery to the myocardium is achieved by injection directly into a coronary artery (which is presently most preferred), a number of approaches can be used to introduce a catheter into the

coronary artery, as is known in the art. By way of illustration, a catheter can be conveniently introduced into a femoral artery and threaded retrograde through the iliac artery and abdominal aorta and into a coronary artery. Alternatively, a catheter can be first introduced into a brachial or carotid artery and threaded retrograde to a coronary artery. The capillary bed of the myocardium can also be reached by retrograde perfusion, e.g., from a catheter placed in the coronary sinus. Such a catheter may also employ a proximal balloon to prevent or reduce anterograde flow as a means of facilitating retrograde perfusion. For delivery to tissues supplied by the peripheral vasculature, catheters can be introduced into arteries supplying such tissues (e.g., femoral arteries in the case of the leg) or may be introduced, by example, as a bolus injection or infusion into the affected tissue. Various combinations of vectors comprising angiogenic genes and catheters or other *in vivo* delivery devices (e.g., other devices capable of introducing a pharmaceutical composition, generally in buffered solution, into a blood vessel or into muscle) can be incorporated into kits for use in accordance with the present invention. Such kits may also incorporate vasoactive agents to enhance gene delivery, and may further include instructions describing their use in accordance with the methods described herein.

Compositions of the present invention can also be administered in conjunction with surgical procedures such as angioplasty and bypass procedures. For treatment of peripheral vascular disease, the compositions likewise can be introduced into one more localized sites, for example by intravascular or direct, e.g., intramuscular, injection. For intravascular delivery, for example, a catheter may be employed to introduce the composition into the femoral artery supplying blood to the affected tissue region. Alternatively, the composition may be injected directly into the muscle affected by disease.

For use by a physician, the compositions will preferably be provided in a dosage form containing an amount of vector of the invention which will be effective, in one or multiple doses, to provide a therapeutic effect. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition, and the level of angiogenesis and/or other effect to be obtained, and other factors.

An effective dose of the viral vectors of this invention will typically be in the range of about $10^5 - 10^{13}$ viral particles, more typically about $10^7 - 10^{10}$ viral particles, depending upon the particular combination used. The relative potency of particular combinations can be readily assessed in preclinical dose-response analyses, as illustrated herein, which typically serve as the basis for selecting doses to be tested in a clinical trial. During the first phase or phases of a clinical trial, it is also typical to evaluate an ascending range of doses in order to select an optimum dose or doses for treatment. The final dose to be administered is determined by the attending clinician. For intravascular delivery, the dose is typically administered in 5 ml or less of a physiologically buffered solution (such as phosphate buffered saline (PBS)) and more typically in 1-3 ml of solution. For direct intramuscular delivery, the final dose is typically administered in about 30 ml or less of physiologically buffered solution (such as, PBS), more typically in 5-20 ml of solution. Typically, administration via intramuscular injection is made using multiple injections, each single injection having a volume of about 2 mL. Thus, for example, in an exemplary embodiment, eight intramuscular injections of 2 mL each are employed to deliver the vector composition.

Animal Models

Important prerequisites for successful studies of cardiovascular gene therapy are (1) constitution of an animal model that is applicable to clinical cardiovascular disease that can provide useful data regarding mechanisms for increased blood flow and/or contractile function, and (2) accurate evaluation of the effects of gene transfer. The porcine model described in the examples herein fulfills these prerequisites. The pig is a particularly suitable model for studying heart diseases of humans because of its relevance to human physiology. The pig heart closely resembles the human heart in the following ways. The pig has a native coronary circulation very similar to that of humans, including the relative lack of native coronary collateral vessels. Additionally, the size of the pig heart, as a percentage of total body weight, is similar to that of the human heart. Further, the pig is a large animal model, therefore allowing more accurate extrapolation of various parameters such as effective vector dosages, toxicity, etc. In contrast, the hearts of animals such as dogs and members of

the murine family have a lot of endogenous collateral vessels. Additionally, relative to total body weight, the size of the dog heart is twice that of the human heart.

The animal model exemplified herein is illustrative of myocardial ischemia. (Since myocardial ischemia can also result in and/or occur in connection with congestive heart failure, this particular model is further relevant to that situation.) In this model, which mimics clinical coronary artery disease (CAD), placement of an ameroid constrictor around the left circumflex (LCx) coronary artery results in gradually complete closure (within 7 days of placement) with minimal infarction (1% of the left ventricle, 4 ± 1% of the LCx bed). For detailed descriptions of this and related models, see the examples herein, and Hammond, et al., WO 96/26742, published 6 Sep. 1996 Hammond et al., USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by *in vivo* Gene Delivery", Roth, et al., Circulation, 82:1778, 1990; Roth, et al., Am. J. Physiol., 235:1-11279, 1987; White, et al., Circ. Res., 71:1490, 1992; Hammond, et al., Cardiol., 23:475, 1994; and Hammond, et al., J. Clin. Invest., 92:2644, 1993.

Another animal model that may be employed herein to demonstrate the gene therapy methods and compositions of the present invention is a model of dilated cardiomyopathy (DCM) such as that observed in clinical congestive heart failure. In this model, continuous rapid ventricular pacing over a period of 3 to 4 weeks induces heart failure which shows similarities with many features of clinical heart failure, including left ventricular dilation with impaired systolic function analogous to regional functional abnormalities seen in heart failure (including those associated with severe coronary artery disease and with non-CAD DCM, such as IDCM). See, e.g., Hammond, et al., WO 98/50079, published 30 Apr 1998.

Other animal models of congestive heart failure include the induction of chronic ventricular dysfunction via intracoronary delivery of microspheres (see e.g. Lavine et al., J Am Coll. Cardiol. 18: 1794-1803 (1991); Blaustein et al., Am. J. Cardio. Path. 5: 32-48 (1994); Sabbah et al. Am. J. Physiol. 260: H1379-H1384 (1991)). As an additional example of ventricular dysfunction, straight occlusion of the left coronary artery in a rat model can induce infarcts and the animals can then be

studied and treated over subsequent days or weeks (see e.g. Pfeffer et al., Circ. Res. 44: 503-512, 1979; Pfeffer et al., Am. J. Physiol. 260: H1406-1414, 1991).

Delivery to an animal's heart of the gene therapy compositions according to the present invention, is generally accomplished by intracoronary delivery as described and illustrated herein. As an initial test of candidate vectors, however, prior to delivery in a large animal model such as pig, we have recently employed a rat model in which we use indirect intracoronary delivery of vector to the myocardium. In that model, delivery is achieved by introduction of a solution comprising the vector (e.g. in phosphate buffered saline (PBS) or HEPES buffered saline) into the chamber of the left ventricle (i.e. by introduction into the lumen of the chamber as opposed to the ventricular wall) after constricting both the pulmonary artery and the distal aorta. Flow from the chamber of the ventricle thus carries the material to be delivered into the coronary arteries since alternative pathways are temporarily blocked. We have used a cross-clamping procedure to constrict the pulmonary artery and aorta (see, e.g., Hajjar, et al., PNAS 95:5251-5256, 1998). We have also employed pretreatment with either histamine, at a concentration of 1-75 micrograms/ml, typically 25 micrograms/ml or sodium nitroprusside (SNP) at a concentration 10-100 micrograms/ml, typically 50 micrograms/ml, as described above, in order to enhance gene transfer via intracoronary delivery. Using these procedures, we have demonstrated very high levels of gene transfer to the myocardium via intracoronary delivery of both adenoviral and AAV vectors. Using rAAV/EGFP, for example, we can achieve transduction of the left ventricle (LV) at levels of about 30% of cells (as measured by fluorescent microscopy, after fixing LV sections in paraformaldehyde and cutting with a cryostat into 8-10 micron sections, and quantifying the percentage of green area using ImagePro Plus software). Gene expression within the myocardium was greatest within the epicardium but significant expression was observed even in the endocardium. After delivering an AAV vector to the myocardium as described, we have demonstrated that gene expression is relatively long-lived (with little if any reduction in expression levels between 30 days and 60 days post-injection). In addition, histological and pathological analyses revealed little or no inflammatory response in the heart and no detectable gene expression in either

the liver or the lung. These methods are likewise expected to be useful for the purposes of evaluating gene therapy vectors comprising at least two angiogenic transgenes in accordance herewith.

With respect to peripheral vascular disease, delivery of angiogenic genes into the peripheral vasculature using gene therapy vectors of the present invention can be examined using, for example, a hind limb ligation model of peripheral ischemia. See, e.g., the femoral artery ligation model described by R.L. Terjung and colleagues (see, for example, Yang, et al., Circ Res, 79(1):62-9 (1996)). As with delivery of angiogenic genes to ischemic myocardium, the delivery of angiogenic genes according to the present invention to the peripheral vasculature and/or associated muscle can be used to overcome effects of peripheral vascular disease.

Thus, these models can be used to determine whether delivery of a vector construct coding for at least two angiogenic peptides or proteins is effective to alleviate the cardiac (or peripheral) dysfunctions associated with these conditions. These models are particularly useful in providing some of the parameters by which to assess the effectiveness of *in vivo* gene therapy for the treatment of congestive heart failure and ventricular remodeling. For additional details on animal models discussed herein, see, e.g., Hammond, et al., WO 96/26742, published 6 Sept. 1996, WO 98/10085, published 12 Mar. 1998, WO 98/50079, published 12 Nov. 1998 and USSN 09/609,080, filed 30 June 2000, entitled "Techniques and Compositions for Treating Cardiovascular Disease by *in vivo* Gene Delivery", each of which has previously been incorporated by reference in its entirety.

EXAMPLES

To assist in understanding the present invention, the following Examples are included which describe preferred embodiments of the compositions and methods disclosed and claimed herein. The examples relating to this invention should not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the ability of one skilled in the art, after reviewing the teachings of the present invention in combination

with the technical skills known in the art, are considered to fall within the scope of the invention as described herein and hereinafter claimed.

Examples 1 and 2 provide exemplary assays useful for evaluating mitogenic and/or angiogenic activity of test proteins or peptides, such as, for example, derivatives of known angiogenic proteins or peptides.

Together, Examples 3-8 illustrate exemplary construction of vectors comprising combinations of angiogenic transgenes according to the present invention and exemplify the use of such vectors in gene therapy.

Example 1: Mitogenic Assays

The mitogenic activity of a protein or peptide, for example a derivative of a known angiogenic protein or peptide, or combinations thereof on cells of human or mammalian origin (e.g. endothelial cells) can be determined by a number of different procedures, including assays in which cell proliferation is measured by growth of cell numbers or by incorporation of radioactive DNA precursors (e.g. thymidine incorporation) or otherwise appropriately labeled DNA precursors (bromo-deoxyuridine incorporation). These and other methods generally used to determine cell proliferation, including those methods where mitogenic activity is assessed *in vivo* (for example by determining the mitotic index of endothelial cells) can be employed within the context of this invention. An illustrative method is as described by Bohlen et al., Proc. Natl. Acad. Sci. USA 81:5364-68, 1994. By way of illustration, bovine aortic arch endothelial cells maintained in stock cultures in the presence of Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics (gentamycin at 50 micrograms/ml and fungizine at 0.25 micrograms/ml) and basic fibroblast growth factor (1-10 nanograms/ml, added every 48h) are passaged weekly at a split ratio of about 1:4. For mitogenic assays, cell monolayers from stock plates (at passages 3-10) are dissociated using trypsin. Cells are then seeded at a density of approximately 10,000-20,000 cells/well in 24-well plates in the presence of DMEM and antibiotics as described above. Samples to be assayed (1-10 microliters), appropriately diluted in DMEM/0.1% bovine serum

albumin), are added six hours after plating of cells and again after 48 hours. After 4 days of culture, endothelial cells are detached from plates with trypsin and counted using a Coulter particle counter and compared to control samples.

Another mitogenic activity assay is provided in Olofsson, B. et al., Proc. Natl. Acad. Sci. USA 93:2576-81, 1996). In an exemplary procedure, second passage human umbilical vein endothelial cells (HUVECs) are plated into 96-well plates (4 X 10^3 cells per well) in M-199 medium supplemented with 10% (vol/vol) fetal bovine serum and incubated for 24 hours. Cell culture conditioned medium containing the test protein or peptide, in the presence of 1-10 micrograms/ml heparin, or purified test protein or peptide is added to the HUVECs, and the cells are stimulated for 48 hours. Fresh cell culture conditioned medium containing [^{3}H] thymidine (Amersham; 10 microcuries/ml) is added to the cells and stimulation is continued for another 6-24 hours. Cells are washed with PBS and trypsinized and the incorporated radioactivity is determined by liquid scintillation counting. The activity of the test protein/peptide is then compared to the activity of the related known angiogenic protein or peptide.

In another exemplary method, bovine capillary endothelial (BCE) cells are seeded into 24-well plates and grown until confluence in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Cells are starved in MEM supplemented with 3% fetal calf serum for 72 hours, after which conditioned medium diluted into serum-free medium is added to the cells and the cells are stimulated for 24 hours. [^{3}H] thymidine is included during the last 4 hours of the stimulation (1 microcuries/ml). Cells are washed with PBS and lysed with NaOH, and incorporated radioactivity is determined by liquid scintillation counting. The activity of the test protein or peptide is compared to that of the related known angiogenic protein or peptide. Bovine fibroblast growth factor may be used as an additional control for mitogenic activity, and may also be used to measure its potentiating activity of the test protein or peptide activity.

Example 2: Angiogenic Assays

The angiogenic activity of substances, such as, for example, derivatives of known angiogenic proteins or peptides or combinations thereof, can be determined using a variety of *in vivo* methods. An exemplary method for demonstrating the angiogenic activity of, for example, an angiogenic protein such as a VEGF-derived protein or peptide is the rabbit corneal pouch assay. In this assay, Elvax (ethylene vinyl acetate) polymer pellets containing approximately 1-1000 nanograms of the test protein/peptide and a constant amount of rabbit serum albumin as carrier is implanted into a surgical incision in the cornea as described in more detail in Phillips and Knighton, Wound Rep. Reg. 3, 533-539, 1995; Gimbrone et al., J. Natl. Canc. Inst. 52:413-27, 1974; Risau, Proc. Natl. Acad. Sci. USA 83:3855-59, 1986). Vascularization of the cornea, induced by the test protein or peptide is then observed over a period of 2 weeks. Semi-quantitative analysis is possible with morphometric and image analysis techniques using photographs of corneas.

Other exemplary methods include the chick chorioallantoic membrane assay (CMA), the corneal pouch assay in rats or mice, the rodent mesenteric-window angiogenesis assay (MWAA), the colorimetric assay in mice, the matrigel implant assay in mice, the rabbit ear chamber angiogenesis assay, the hamster cheek pouch assay, the Hunt-Schilling chamber model and the rat sponge implant model, as well as numerous other assay methods to assess the formation of new blood vessels which have been described in the literature. See, e.g., Schreiber, et al., Science, 232(4755:1250-3 (1986); Norrby, et al., Virchows Arch. B. Cell Pathol., 52:195-206 (1986); Andrade, et al., Br. J. Exp. Pathol. 68(6:755-66 (1987); Peek, et al., Exp. Pathol., 34(1):35-40 (1988); Spalwinski, et al., Methods Find Exp. Clin. Pharmacol., 10(4):221-6 (1988); Lee, et al., Cancer Chemother. Pharmacol., 26(6):461-3 (1990); Norrby, et al., Microvasc. Res. 39(3):341-8 (1990); Lebel, et al., Int. J. Exp. Pathol., 72(2):111-8 (1991); and Phillips, et al, *In Vivo*, 8(6):961-5 (1994), describing various assays and/or the use thereof to test for angiogenesis.

Example 3: Exemplary Angiogenic Transgenes

By way of illustrating the construction and use of combinations of angiogenic transgenes according to the present invention we selected an exemplary pair of angiogenic genes to be combined into a single gene delivery vector. As described above, for use in the present invention, such angiogenic genes can be combined with each other and with regulatory sequences in a number of different ways. Illustrative examples are provided below.

Example 3.1: Illustrative Example Employing Two Different Angiogenic Genes, each Regulated by a Constitutive Promoter

As an initial illustration, we used an exemplary FGF gene and an exemplary VEGF gene, each regulated by a constitutive promoter, which were then combined into a single expression vector. As an exemplary FGF gene, we used a fibroblast growth factor-4 (FGF-4) gene having an N-terminal deletion of the first 36 amino acids of the mature FGF-4 protein and including the native signal peptide. This FGF-4 fragment, also known as K140, is described in detail by Basilico, et al. in U.S. Patent 5,459,250 (17 Oct 1995), which patent is hereby incorporated by reference, in its entirety.

As an exemplary VEGF gene, a variant (or derivative) of VEGF-145, lacking Domain II of exon 6, was employed (abbreviated as VEGF-145v2 below). This VEGF-145 variant is described in detail by Neufeld, et al., in pending U.S. Patent application number 09/037,983, filed 11 Mar 1998 and hereby incorporated by reference, in its entirety.

As also described above, various regulatory sequences may be employed in conjunction with the combination of angiogenic transgenes to effect and/or control transcription thereof. As a first illustrative example, CMV promoters were operably linked to each of the above-referenced FGF and VEGF transgenes. Similarly, polyadenylation sequences were employed at the 3' ends of each transgene to facilitate termination of transcription. Standard recombination techniques were used to construct the exemplary expression cassettes, each cassette comprising a separate transcriptional unit including an angiogenic gene and associated regulatory sequences.

The two expression cassettes were then inserted into a gene delivery vector, as described further below. The resulting dual FGF/VEGF vector comprised a viral sequence with an insert of approximately 4.5 kb comprising, in order in the 5' to 3' direction, a CMV promoter, an intron sequence, the exemplary FGF fragment, a polyadenylation sequence (e.g. a polyadenylation sequence derived from the bovine growth hormone gene), a second CMV promoter, the exemplary VEGF fragment, and an SV40 polyadenylation/intron sequence. Thus, in this example the expression cassettes provided the transgenes as two separate but adjacent transcriptional units within the final vector construct.

Example 3.2: Illustrative Example Employing Different Angiogenic Transgenes Regulated by Different Promoters

As a second illustration, each angiogenic transgene is operably linked to a different promoter. This configuration is useful, for example, to provide increased flexibility and/or control over gene expression and/or to further reduce the frequency of homologous recombination during repeat propagation of the vector. In this illustrative example, the two different promoters can be independently selected and can be constitutive or inducible. By way of example, a first angiogenic gene is operably linked to a CMV promoter and a second angiogenic gene is linked to a different promoter such as an RSV or other constitutive promoter, or alternatively, an inducible promoter. The same procedures described above can be used to construct these alternative expression cassettes, resulting in a final vector insert having two different promoters, each operably linked to an angiogenic gene (eg. $P_1(RSV)\rightarrow A_1\rightarrow P_2(CMV)\rightarrow A_2$ or, $P_1(CMV)\rightarrow A_1\rightarrow P_2(RSV)\rightarrow A_2$, where P represents a promoter and A represents a transgene encoding an angiogenic protein or peptide including any necessary or desired regulatory sequences, such as polyadenylation sequences and where the genes are arranged in an adjacent, head to tail configuration).

In exemplary constructs, the FGF-4 fragment and the VEGF-145 variant referred to above are introduced into the previously described constructs such that in

one construct FGF-4 is driven by the CMV promoter and the VEGF variant by the RSV promoter, and in the other construct these are reversed.

Example 3.3: Illustrative Example Employing Different Angiogenic Proteins Regulated by the Same Promoter

As discussed previously herein, the two transgenes can alternatively be fused into a single transcriptional unit under the control of a single promoter, which can be designed to yield a single protein or two separate protein products. The promoter can be a constitutive promoter such as CMV or RSV or it can be an inducible promoter as discussed above. Additional promoters include, but are not limited to, tissue- and cell- specific promoters such as members of the cardiomyocyte-specific promoter family or other heart specific promoters (where the heart is the target for treatment).

By way of illustration, a single promoter can be used to yield two separate angiogenic proteins. Preferably, an internal ribosome entry site (IRES) sequence is provided between the sequences, most optimally between about 100 and 500 nucleotides after the termination codon of the first transgene (see eg. Attal J., et al., Genet. Anal. 15(3-5): 161-5, 1999). An exemplary construct would thus comprise the sequence: $P \rightarrow A_1 \rightarrow I \rightarrow A_2$, where P represents a promoter, A_1 represents a transgene encoding a first angiogenic protein or peptide, I represents an IRES sequence, and A_2 represents a transgene encoding a second angiogenic protein or peptide. This arrangement can be used to generate a single transcript which can be modified to improve levels of production of the second "downstream" protein of the pair. IRES sequences are typically cis-acting elements which recruit the small ribosomal subunits to an internal initiator codon in the mRNA with the aid of cellular trans-acting factors. A number of different IRES sequences are known and have been shown to be useful in polycistronic transcription units for efficient transcription of multiple transgenes.

Alternatively, a single promoter may be employed to yield a single translation product. As discussed previously and by way of example, the two angiogenic transgenes can be effectively transcriptionally and translationally fused, i.e., fused into a single gene, translation of which results in a single bifunctional peptide (or a

single polypeptide including a post-translation signaling sequence that results in post-translation cleavage into two separate, functional peptides). Preferably, the transgenes encoding a bifunctional peptide include a polynucleotide spacer sequence therebetween containing no stop codons. In a preferred embodiment, the spacer is configured to encode a short, flexible polypeptide sequence (or "flexon"), for example, to facilitate optimal, independent folding and activity of the two angiogenic peptides on either side thereof.

Example 4: Exemplary Construction of a Gene Delivery Vector

As discussed above, various gene delivery vectors known to those of skill in the art can be employed in accordance with the present invention. For the purpose of illustration, we have chosen to use a human type 5 adenoviral vector (Ad5) that has been rendered replication defective by deletion of the essential E1A and E1B genes.

Standard recombination and subcloning techniques were employed to construct plasmids comprising each expression cassette, which were then combined, by subcloning, into a single plasmid comprising the two exemplary angiogenic transgenes and associated regulatory sequences in a tandem, head-to-tail orientation. This plasmid was then packaged into an adenoviral vector using standard rescue recombination techniques, to yield the final recombinant adenovirus construct, Ad5FGF4f-VEGF145v2.

The FGF-4 fragment (FGF4f) expression cassette was constructed using a CMV promoter (with intron) and bovine growth hormone polyadenylation sequence as illustrative regulatory sequences. For the VEGF145v2 expression cassette, a CMV promoter (without intron) and SV40 intron polyadenylation sequence were the exemplary regulatory sequences.

As discussed above, numerous alternatives exist in selecting regulatory sequences for use herein. By way of illustration, an IRES sequence may be employed between the two angiogenic transgenes with a single promoter, such as a CMV or RSV promoter, upstream of the first transgene and driving transcription of the two transgenes. The resulting single transcription product, comprising an IRES sequences

between the two transgenes, is then translated into two distinct angiogenic proteins or peptides.

Adenovirus 5 (Ad5) was selected as an initial illustrative gene delivery vector. As stated above, the replication-deficient adenoviral vector exemplified herein was constructed by the rescue recombination method. (See, e.g., Graham, Virology 163:614-617, 1988). Briefly, the exemplary dual recombinant plasmid, pAd5FGF4f-VEGF145v2, was co-transferred (by lipofection) into 293 cells with plasmid JM17 (pJM17) which contains the entire human adenoviral 5 genome with an additional 4.3 kb insert making pJM17 too large to be encapsidated. Homologous rescue recombination resulted in adenoviral vectors containing the FGF4f and VEGF145v2 transgenes in place of the Ad5 E1A/E1B sequences, which are necessary for viral replication.

The system used to generate such recombinant adenoviruses imposes a packing limit of about 5kb for transgene inserts. The dual recombinant, Ad5FGF4f-VEGF145v2 insert is approximately 4.5 kb, well within these packaging constraints. Although the recombinants are non-replicative in mammalian cells, they can propagate in 293 cells which have been transformed with the E1A/E1B sequences and thus, provide these essential gene products in a trans-acting fashion. The transfected 293 cells are monitored for evidence of cytopathic effect which usually occurs 10-14 days after transfection. To identify successful recombinants, viral DNA can be isolated from virus particles (from the cell supernatant) and then analyzed by PCR using a primer specific for the angiogenic transgene sequence and a primer specific for the promoter and/or other specific sequence located in the viral construct (see, e.g., Biotechniques 15:868-72, 1993).

Successful recombinants were plaque purified twice, using standard procedures. Viral stocks were propagated in 293 cells to titers typically ranging between 10^8 and 10^{13} viral particles. Cells are typically infected at 80% confluence and harvested at 36-48 hours. After freeze-thaw cycles the cellular debris is pelleted by standard centrifugation and the virus further purified by double CsCl gradient ultracentrifugation (discontinuous 1.33/1.45 CsCl gradient; cesium prepared in 5 mM

Tris, 1 mM EDTA (pH 7.8); 90,000 x g (2 hr), 105,000 x g (18 hr)). Prior to *in vivo* injection, the viral stocks can be desalted by gel filtration through Sepharose columns such as G25 Sephadex. The resulting viral stock preferably has a final viral titer approximately in the 10^8-10^{13} viral particles range.

As additional examples of angiogenic compositions of the present invention, constructs have been generated comprising various illustrative combinations of angiogenic genes and operably-linked regulatory sequences, in various orientations. As described above, combinations including angiogenic genes from different families can be advantageous in that multiple different functionalities can be brought together, that the combinations can be synergistic, and that individual patients may be more responsive to one angiogenic gene product than another. Illustrations of such constructs employing an FGF-4 fragment (FGF4f) and a VEGF-145 variant (VEGF145v2), as described above, include the following: CMV-FGF4f-IRES-VEGF145v2; CMV-FGF4f-RSV-VEGF145v2; CMV-FGF4f-Hef1alpha/HTLV-VEGF145v2; RSV-FGF4f-CMV-VEGF145v2; Hef1alpha/HTLV-FGF4f-CMV-VEGF145v2.

These and other compositions can be readily generated using techniques as described and illustrated herein, and their angiogenic capability can be tested as described above. Angiogenic compositions including these and other constructs generated according to the teachings of the present invention can then be examined to confirm their potency in a suitable animal model of cardiovascular disease, as described above. By way of illustration, compositions of the present invention can be examined in a large animal model of ischemic heart disease as described below.

Example 4.1: Further Illustrative Examples of Construction of Gene Delivery Vectors Comprising Alternative Transgene Configurations

Employing techniques described herein, further illustrative examples of gene delivery vectors comprising alternative transgene configurations were constructed. These included the following: CMV-FGF4f-RSV-VEGF145v2; CMV-FGF4f-Hef1-alpha-VEGF145v2; and CMV-FGF4f-IRES-VEGF145v2. Each of these transgene

combinations was inserted into a human adenoviral vector (Ad5) using standard homologous recombination techniques as described herein and known in the art. Transgene expression was confirmed using standard molecular biology techniques. In this case, we used ELISAs and western blots for each construct. In addition, we performed mitogenic assays as described in Example 1 and confirmed that each construct had a mitogenic activity equivalent to or greater than the mitogenic activity of the CMV-FGF4f-CMV-VEGF145v2 construct described in Example 4.

Example 5: Porcine Ischemia Model For Angiogenesis

As an illustration of an animal model that can be used in a gene therapy context, we have used a porcine ischemia model (as described, for example, by Hammond, et al., in U.S. Patent No. 5,792,453, 11 Aug 1998). This large animal disease model is preferable as it provides better predictability of human therapy than many other animal models.

In this example, domestic pigs (35-40 kg) underwent a left thoracotomy, under sterile conditions, for instrumentation. (Hammond, et al. J Clin Invest. 92:2644-52 (1993); Roth, et al. J. Clin. Invest. 91:939-49, 1993). Catheters were placed in the left atrium, pulmonary artery and aorta, providing a means to measure regional blood flow, and to monitor pressures. Wires are sutured on the left atrium to permit ECG recording and atrial pacing. Finally, an ameroid constrictor (ameroid), a metal ring including an ameroid substance, was placed around the proximal left circumflex coronary artery (LCx) (Hammond et al. J. Clin. Invest. 92:2644-52 (1993)). The ameroid material is hygroscopic and slowly swells, leading gradually to complete closure of the artery within about ten days after placement, with minimal infarction due to the development of collateral blood vessels. Myocardial function and blood flow are normal at rest in the region previously perfused by the occluded artery (the ischemic region), but blood flow is insufficient to prevent ischemia when myocardial oxygen demands are increased. Collateral vessel development is complete within twenty-one days of ameroid placement and remains unchanged for at least four months (Roth, et al., Am. J. Physiol. 253 H1279-H1288 1987). The animals have no

ischemia at rest, but develop ischemia during activity or atrial pacing. Atrial pacing at 200 bpm was used to induce myocardial ischemia.

Example 6: Illustrative Gene Therapy Using Compositions of the Present Invention

As stated above, a porcine ischemia model of cardiovascular disease was selected to illustrate applicability of the present invention to the treatment of myocardial ischemia as it is particularly predictive of human therapy. Thus, once ischemia was established in the animal model, the model was used to demonstrate the gene therapy methods of the present invention.

Approximately 5 weeks after ameroid placement at a time when collateral vessel development and pacing-induced dysfunction are stable (Roth, et al. Am J Physiol 253:1-11279-1288, 1987, and Roth, et al. Circulation 82:1778-89), animals underwent studies to define pacing-induced regional heart function and blood flow. Thereafter, either the dual recombinant adenovirus, Ad5FGF4f-VEGF145v2, or vehicle alone was delivered to the pigs. By way of illustration, intracoronary injection was employed for delivery of the adenovirus (or vehicle alone). For such intracoronary delivery, animals were anesthetized and a 5F arterial sheath was placed into the carotid artery. A 5F multipurpose (A1, end hole only) coronary catheter was inserted through the sheath and into the coronary arteries. Closure of the ameroid was confirmed in all animals by contrast injection into the left coronary artery. The catheter tip was then placed at least about 1 cm within the arterial lumen so that little if any material would be lost to the proximal aorta during injection. Five ml of the adenovirus expressing the two angiogenic transgenes were delivered by slowly injecting 3.0 ml into the left and 2.0 ml into the right coronary arteries using an injection rate of about 2 ml/min. Four different dose groups were studied: 10^7, 10^8, 10^9 and 10^{10} viral particles.

Approximately two weeks following gene transfer, studies to define pacing-induced regional function and blood flow were repeated, animals were then sacrificed and heart, lung, and liver tissues were collected for examination.

Example 7: Evaluation of Angiogenic Gene Therapy in Treated Tissue

Various methods can be used to assess the effects of angiogenic gene therapy on cardiac function. As an illustration of parameters reflecting cardiac function, we evaluated two-dimensional and M-mode images obtained from a right parasternal approach at the papillary muscle level (Hewlett Packard Sonos 1000). Conscious animals were studied while suspended in a comfortable hammock to minimize body movement. Images were recorded with animals in a basal state and again during left atrial pacing (200 bpm). These studies were performed one day prior to gene transfer and repeated approximately fourteen days later. Rate-pressure products and left atrial pressures were similar in both groups before and after gene transfer, indicating similar myocardial oxygen demands and loading conditions. Echocardiographic measurements were made using standardized criteria (Sahn, et al., Circulation 58:1072-1083 1978). End-diastolic wall thickness (EDWTh) and end-systolic wall thickness (ESWTh) are measured from 5 continuous beats and averaged. Percent wall thickening (%WTh) is then calculated [(EDWTh-ESWTh)/EDWTh] X 100. Data is preferably analyzed without knowledge of which treatment the animals had received. To demonstrate reproducibility of echocardiographic measurements, animals are preferably imaged on two consecutive days, showing high correlation (r^2=0.90; p=0.005).

The reproducibility of echocardiographic measurements has previously been documented (Hammond, et al., J. Clin. Invest. 92:2644-2652 1993; and Hammond, et al., Amer. Coll. Cardiol. 23:475-482 1994). Additionally, the percent decrease in function measured by trans-thoracic echocardiography and sonomicrometry, in this model are similar, confirming the accuracy of echocardiography for evaluation of ischemic dysfunction (Hammond, et al., J. Clin. Invest. 92:2644-2652 1993; and Hammond, et al., Amer. Coll. Cardiol. 23:475-482 1994).

Contrast echocardiography confirmed that regional perfusion (as measured by maximum contrast echo enhancement), was substantially increased in the treated animals relative to control animals. Briefly, left atrial injection of contrast material (microaggregates of galactose) increase the echogenicity (whiteness) of the

echocardiographic image. The microaggregates distribute into the coronary arteries and myocardial walls in a manner that is proportional to blood flow. The peak intensity of contrast enhancement is correlated with myocardial blood flow as measured by microspheres (Skyba, et al., Circulation 90:1513-1521 1994).

Approximately five weeks after ameroid placement, well after ameroid closure, but before gene transfer, contrast echocardiographic studies were performed during atrial pacing (200 bpm), by injecting contrast material (e.g., Levovist) into the left atrium. Studies were repeated approximately fourteen days after gene transfer. Peak contrast intensity was measured from the video images using a computer-based video analysis program (Color Vue II, Nova Microsonics, Indianapolis, Indiana) that provided an objective measure of video intensity. Data are expressed as the ratio of the peak video intensity in the ischemic region (LCx bed) divided by the peak video intensity in the interventricular septum (IVS), a region receiving normal blood flow through the unoccluded left anterior descending (LAD) coronary artery. The differences in regional blood flow during atrial pacing measured by contrast echocardiography were similar to the differences measured by microspheres in this same model (Hammond, et al., J. Clin. Invest. 92:2644-2652 1993; and Hammond, et al., Amer. Coll. Cardiol. 23:475-482 1994), documenting the accuracy of echocardiography for the evaluation of regional myocardial blood flow. Myocardial blood flow can also be quantitated by methods such as thallium imaging, whereby the heart is perfused with the radionuclide thallium as described by Braunwald in Heart Disease, 4th ed., pp.276-311 (Saunders, Philadelphia, 1992). Cells in the heart have an avidity for thallium and therefore, the increased thallium uptake is correlated with increased blood flow. All individuals performing and analyzing echocardiographic measurements of regional function and perfusion were blinded to the treatment regimen (i.e., test article and dose).

Example 8: Results of *In Vivo* Gene Therapy

Results of the determination of percent wall thickening in the collateral-dependent left circumflex (LCx) bed, based on echocardiographic measurements, is presented in Table 1. Percent wall thickening before and fourteen days after intracoronary gene transfer are provided for each virus dose tested and for the vehicle

alone control. As can be seen from Table 1, the Ad5FGF4f-VEGF145v2 vector, given in doses ranging from as low as 1 x 10^7 up to 1 x 10^{10} virus particles, resulted in improvements of regional heart function, as illustrated by percent wall thickening. The results obtained using 1 x 10^7 viral particles of this dual recombinant vector were similar to results observed with approximately 1 x 10^{10} viral particles comprising FGF-4 alone. The results thus demonstrate that combinations of angiogenic genes prepared and delivered as described herein were highly effective in improving cardiac function in a large animal model of cardiovascular disease. In addition these results indicate that the adenovirus containing two angiogenic transgenes appears to be much more effective than a single angiogenic transgene construct (FGF-4 alone).

TABLE 1

Percent Wall Thickening During Atrial Pacing at 200 BPM

Group		**% Wall Thickening (LCx)**	
A. Dose	**B.** N	**Pre treatment**	**14 days post treatment**
Ad5FGF-4f-VEGF145v2			
1 x 10^7	3	22.3 ± 1.5	49.3 ± 5.0
1 x 10^8	3	23.7 ± 6.7	50.7 ± 13.6
1 x 10^9	2	23.5 ± 2.1	53.5 ± 3.5
1 x 10^{10}	2	27.5 ± 0.7	56.5 ± 3.5
Vehicle (Control)			
5 ml	2	27 ± 5.6	34 ± 7.1

Claims:

1. A method for treating cardiovascular disease in a patient comprising delivering to the patient at least two transgenes encoding angiogenic proteins or peptides, which transgenes are delivered *in vivo* in at least one gene delivery vector.

2. The method of claim 1, wherein said vector is a viral vector.

3. The method of claim 2, wherein said vector is a replication-deficient viral vector.

4. The method of claim 3, wherein said vector is an adenovirus vector.

5. The method of claim 3, wherein said vector is an adeno-associated viral vector.

6. The method of claim 3, wherein about 10^5 to about 10^{13} viral particles are delivered *in vivo*.

7. The method of claim 3, wherein about 10^7 to about 10^{10} viral particles are delivered *in vivo*.

8. The method of claim 1, wherein expression of at least one of said transgenes is driven by a constitutive promoter which is contained in the vector.

9. The method of claim 1, wherein expression of at least one of said transgenes is driven by a CMV promoter which is contained in the vector.

10. The method of claim 1, wherein expression of at least one of said transgenes is driven by a tissue-specific promoter which is contained in the vector.

11. The method of claim 10, wherein expression of at least one of said transgenes is driven by a cardiomyocyte-specific promoter which is contained in the vector.

12. The method of claim 11, wherein said cardiomyocyte-specific promoter is selected from the group consisting of a myosin light chain promoter and a myosin heavy chain promoter.

13. The method of claim 1, wherein the angiogenic proteins or peptides encoded by the at least two transgenes are independently selected from the group of angiogenic protein families consisting of fibroblast growth factors, vascular endothelial growth factors, platelet-derived growth factors, insulin-like growth factors, hypoxia-inducible factors, angiogenic zinc-finger proteins, and angiopoietins.

14. The method of claim 13, wherein the angiogenic proteins or peptides encoded by the at least two transgenes are selected from different angiogenic protein families.

15. The method of claim 14, wherein one of said angiogenic proteins or peptides is a fibroblast growth factor and the other of said angiogenic proteins or peptides is a vascular endothelial growth factor.

16. The method of claim 15, wherein said fibroblast growth factor is an FGF-4.

17. The method of claim 16, wherein said fibroblast growth factor is FGF-4f.

18. The method of claim 15, wherein said vascular endothelial growth factor is a VEGF-A.

19. The method of claim 18, wherein said VEGF-A is selected from the group consisting of VEGF-145, VEGF-145v2 and VEGF-138.

20. The method of claim 15, wherein one of said angiogenic proteins or peptides is FGF4f and the other of said angiogenic proteins or peptides is VEGF-145v2.

21. The method of claim 14, wherein one of said angiogenic proteins or peptides is a fibroblast growth factor or a vascular endothelial growth factor and the other of said angiogenic proteins or peptides is an insulin-like growth factor.

22. The method of claim 21, wherein said insulin-like growth factor is IGF-1.

23. The method of claim 1, wherein said vector comprises transgenes encoding a fibroblast growth factor, a vascular endothelial growth factor and an insulin-like growth factor.

24. The method of claim 1, wherein the vector is introduced into a tissue by anterograde perfusion from a catheter placed into a conduit delivering blood to the tissue.

25. The method of claim 1, wherein the vector is introduced into a tissue by retrograde perfusion from a catheter placed into a conduit receiving blood from the tissue.

26. The method of claim 1, wherein the cardiovascular disease is a heart disease and the vector is delivered to the myocardium of the patient.

27. The method of claim 26, wherein the vector is introduced into at least one coronary artery, whereby the transgenes are delivered to the myocardium and expressed.

28. The method of claim 1, wherein the vector is introduced into a saphenous vein graft and/or an internal mammary artery graft supplying blood to the myocardium.

29. The method of claim 1, wherein the vector is introduced by retrograde perfusion from a catheter placed into a conduit receiving blood from the myocardium.

30. The method of claim 1, wherein the cardiovascular disease is peripheral vascular disease.

31. The method of claim 30, wherein the vector is introduced into an ischemic peripheral tissue by perfusion into an artery supplying blood to the tissue.

32. The method of claim 30, wherein the vector is introduced into an ischemic peripheral tissue by intramuscular injection.

33. The method of claim 24, further comprising introducing a solution which comprises perfusing a vasoactive agent into the conduit prior to or coincident with delivery of the vector.

34. The method of claim 33, wherein the vasoactive agent is selected from the group consisting of histamine, a histamine agonist, a VEGF protein and sodium nitroprusside.

35. A kit for gene therapy comprising a composition selected from the group consisting of: (i) a composition which comprises a single vector comprising at least two transgenes encoding angiogenic proteins or peptides, and (ii) a composition which comprises at least two vectors, each vector comprising a transgene encoding a different angiogenic protein or peptide.

36. A kit of claim 35, further comprising a device for introducing the composition into a blood vessel or tissue *in vivo*.

37. A kit of claim 36, wherein the device is a catheter.

38. A kit of claim 35, further comprising a solution which comprises a vasoactive agent.

39. A kit of claim 38, wherein the vasoactive agent is selected from the group consisting of histamine, a histamine agonist, a VEGF protein and sodium nitroprusside.

40. A composition comprising a vector for *in vivo* gene delivery, wherein said vector comprises at least two transgenes encoding angiogenic proteins or peptides.

41. A composition of claim 40, wherein said vector is a viral vector.

42. A composition of claim 40, wherein said vector is a replication-deficient viral vector.

43. A composition of claim 42, wherein said vector is an adenovirus vector.

44. A composition of claim 43, wherein said vector is a an adeno-associated viral vector.

45. A composition of claim 40, wherein the vector comprises two operons, each of which comprises an angiogenic gene operably linked to one or more transcriptional regulatory sequences.

46. A composition of claim 45, wherein said operons are in a tandem orientation relative to each other in the vector.

47. A composition of claim 40, wherein the vector comprises an operon which comprises a promoter operably linked to two adjacent angiogenic gene coding sequences.

48. A composition of claim 47, wherein transcription from said promoter results in a single transcript comprising an IRES sequence, whereby said transcript is translated into two angiogenic proteins or peptides.

49. A composition of claim 47, wherein transcription from said promoter results in a single transcript containing a single open reading frame.

50. A composition of claim 49, wherein transcription and translation of said transcript result in the production of a bi-functional polypeptide comprising the amino acid sequences of two different angiogenic proteins or peptides.

51. A composition of claim 45, wherein the vector comprises two promoters, each of which promotes the expression of a different angiogenic transgene.

52. A composition of claim 51, wherein the two promoters are different from each other.

53. A composition of claim 51, wherein at least one of said promoters is a constitutive promoter.

54. A composition of claim 51, wherein at least one of said promoters is a CMV promoter.

55. A composition of claim 51, wherein at least one of said promoters is a tissue-specific promoter.

56. A composition of claim 51, wherein at least one of said promoters is a cardiomyocyte-specific promoter.

57. A composition of claim 51, wherein said cardiomyocyte-specific promoter is selected from the group consisting of a myosin light chain promoter and a myosin heavy chain promoter.

58. A composition of claim 40, wherein the angiogenic proteins or peptides encoded by the at least two transgenes are independently selected from the group of angiogenic protein families consisting of fibroblast growth factors, vascular endothelial growth factors, platelet-derived growth factors, insulin-like growth factors, hypoxia-inducible factors, angiogenic zinc-finger proteins and angiopoietins.

59. A composition of claim 58, wherein the angiogenic proteins or peptides encoded by the at least two transgenes are selected from different angiogenic protein families.

60. A composition of claim 59, wherein one of said angiogenic proteins or peptides is a fibroblast growth factor and the other of said angiogenic proteins or peptides is a vascular endothelial growth factor.

61. A composition of claim 60, wherein said fibroblast growth factor is an FGF-4.

62. A composition of claim 61, wherein said fibroblast growth factor is FGF-4f.

63. A composition of claim 60, wherein said vascular endothelial growth factor is a VEGF-A.

64. A composition of claim 63, wherein said VEGF-A is selected from the group consisting of VEGF-145, VEGF-145v2 and VEGF-138.

65. A composition of claim 60, wherein one of said angiogenic proteins or peptides is FGF-4f and the other of said angiogenic proteins or peptides is VEGF-145v2.

66. A composition of claim 59, wherein one of said angiogenic proteins or peptides is a fibroblast growth factor or a vascular endothelial growth factor and the other of said angiogenic proteins or peptides is an insulin-like growth factor.

67. A composition of claim 66, wherein said insulin-like growth factor is IGF-1.

68. A composition of claim 66, wherein one of said angiogenic proteins or peptides is an FGF-4 and the other of said angiogenic proteins or peptides is IGF-1.

69. A composition of claim 67, wherein said FGF-4 is FGF-4f.

70. A composition of claim 66, wherein one of said angiogenic proteins or peptides is a VEGF-A and the other of said angiogenic proteins or peptides is IGF-1.

71. A composition of claim 70, wherein said VEGF-A is selected from the group consisting of VEGF-145, VEGF-145v2 and VEGF-138.

72. A composition of claim 40, wherein at least one of said angiogenic proteins or peptides comprises a signal peptide.

73. A composition of claim 40, further comprising a pharmaceutical excipient.